

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

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VOLUME XXVII 1933

PART II, pp. 1061 to end

CAMBRIDGE
AT THE UNIVERSITY PRESS

1933

5805-33

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CXL. A DEFICIENCY DISORDER INDUCED IN SUCKLING YOUNG RATS BRED ON A PURIFIED SYNTHETIC DIET WITH "GLAXO CASEIN" (CASEINOGEN) AS SOLE SOURCE OF PROTEIN.

By LESLIE WILLIAM MAPSON.

From the Biochemical Laboratory, Cambridge.

(Received June 27th, 1933.)

IN a former communication [Mapson, 1932] the stimulating effect on growth of small amounts of mammalian liver fed as supplement to a purified basal synthetic diet containing all hitherto known dietary principles was demonstrated. It was shown that young rats when transferred from the stock diet to the synthetic ration and fed in addition on small amounts of fresh ox-liver showed a greatly accelerated growth over control animals. Evidence was submitted of the non-identity of this growth-promoting factor with any of the better-known vitamins. This growth-promoting principle was provisionally named physin. During the course of this work the anomalous behaviour of a small number of litters was observed.

The addition of small amounts of liver to the diet failed to give the usual acceleration in growth seen in the majority of the litters. These variable results were in contradistinction to the very uniform and consistent responses obtained when liver was fed not directly to the offspring but by transmission from the parent animal.

These results were explicable on the assumption of a seasonal variation in the content of physin in the stock dietary. This assumption was supported by the fact that control offspring from litters showing little or no effect of the liver feeding displayed an absolute growth rate of a higher magnitude than that of control animals from litters in which the acceleration effect of the liver feeding was apparent. Moreover, the growth rate of control animals in the first group was approximately the growth rate given by the liver-fed offspring of the second group.

An attempt has been made in the more recent work to eliminate as far as possible the effect of a stock dietary and to render the young animals more uniformly deficient in this growth-promoting factor. The further possibility had to be borne in mind that the actual synthetic diet originally used was not entirely deficient in physin. The existence of such a dietary principle had been demonstrated in the former work merely by its stimulating effect on growth, it was hoped by using a completely deficient diet, that animals might show definite pathological symptoms of such a deficiency.

With the latter consideration in mind the synthetic diet used in the former work has been modified in various respects. The results of these experiments here recorded indicate in a striking manner the nutritional differences existing

between various caseinogen preparations. A definite deficiency disorder has been disclosed by a modification of the original synthetic diet by the substitution of a different brand of caseinogen in the ration.

This deficiency has been cured by the addition of active curative extracts containing physin to the basal ration.

EXPERIMENTAL.

It was considered that even though modifications of the former synthetic diet might not yield a ration entirely deficient in physin, and might therefore still sustain some growth in the young animal, such low physin-containing diets might yet be inadequate for normal breeding and lactation. Hence offspring from such parents might be expected to be relatively more deficient in physin and more uniform in this deficiency than experimental animals derived directly from the stock diet used in this laboratory.

The experimental procedure has thus been to breed animals on modifications of the original basal diet, and a twofold study of the lactating powers of the females and growth rate of the offspring on such diets has been attempted.

All the animals used were black and white rats from the stock strain bred in this laboratory. The young animals were separated at weaning from the stock diet at an age of approximately 4-5 weeks. Their weight at this age ranged from 40 to 60 g.

At this stage they were placed on the synthetic basal ration. All the synthetic diets were fed *ad libitum*.

Some 16-20 weeks were allowed to elapse before mating of the animals occurred. During gestation and lactation only the synthetic diets were used.

The principal modification of the original synthetic diet has been the substitution of "Glaxo casein" (caseinogen) for "light white casein B.D.H." (Na caseinate). The composition of the synthetic diets used is shown in Table I.

Table I. *Composition of diets (parts by wt.).*

Diet	E	E'	B	F
Protein				
"Glaxo casein"	—	—	—	23
"Light white casein"	23	23	23	—
Rice starch	40	40	46	40
Sugar	17	17	17	17
Arachis oil	15	15	15	15
Wheat embryo	8	8	2	8
Dried yeast	8	8	8	8
Cod-liver oil	2.5	2.5	2.5	2.5
Salt mixture	5	5	5	5
Manganese sulphate (anhydrous)	1 mg.	10 mg.	1 mg.	1 mg.

Cod-liver oil was incorporated in the basal diets. The diets were made up only in small quantities at a time so as to obviate any possibility of deterioration of any of the vitamins included.

The stock diet used in this laboratory consists essentially of mixed corn, wheat embryo, dried yeast, bread and fresh milk daily. The salt mixture used in the synthetic diets was a modification of that used by Osborne and Mendel; the complete composition was given in the former communication [Mapson, 1932].

Breeding on Diet E.

The general results with breeding on the original synthetic diet (Diet E) have been described fully in the former publication. Failure of lactation is common in from 50 to 70 % of the cases studied. This failure occurs at the very outset of the lactation period. The parents neglect their offspring from birth, and in many cases they kill and eat them. Out of some 60 litters bred on this diet no single case is on record in which the mother started to feed her offspring and then eventually failed to rear them. Those litters in which suckling was commenced were completely successful during all the later stages. The young weaned on this diet exhibited an exceptionally good growth rate. No pathological symptoms were seen in any of the litters in which suckling was commenced by the parent. The average weights of the offspring on this diet were: 1st week, 12-14 g.; 14 days, 23-26 g.; 21 days, 35 g.; 28 days, 45-50 g.

Substitution of "light white casein" by "Glaxo casein." Diet F.

This diet differed from Diet E only in the substitution of "Glaxo casein" (caseinogen) for "light white casein B.D.H." (Na caseinate). Breeding occurred as in Diet E and here again a large percentage of cases showed the typical immediate failure of suckling at birth. At this point, however, the resemblance between the two diets ceased, for of the mothers which succeeded in starting to feed their young only a small percentage were successful in rearing any of their offspring. With these latter litters suckling was successful up to a period varying from 14 to 28 days, when the offspring, very much undersized in weight compared to offspring bred on Diet E, showed a definite failure to grow. The onset of the symptoms in a few cases was delayed until after weaning, but in the majority of cases definite pathological symptoms were evident at approximately the 24th day. The baby animals developed sparse greasy coats, looked weak and were seen to have less than normal activity. The occurrence of these symptoms was followed rapidly by the appearance of exhaustion, and finally death ensued within a few hours. In many of the litters before this extreme condition was reached, a vitamin deficiency behaviour was shown by the more lively animals, these attaching themselves to their more moribund companions and frequently eating their entire viscera.

Post mortem examination of the young rats showed no macroscopic signs of pathological change or of infection in any of the organs, except for the extraordinary absence of fat anywhere in the body.

To all outward appearances, in those litters in which the symptoms developed prior to weaning, the mother continued suckling her young right up to the stage of exhaustion.

In the case of a small number of the litters studied some of the young offspring survived without addition of any supplement to the basal ration. That the synthetic Diet F is not completely deficient in this principle seems possible from the fact that young rats saved from death by the feeding of effective supplements were, after some 10-15 days, able to maintain themselves and grow on the basal diet alone. The explanation adopted here is that the basal ration F, though adequate for normal requirements during growth, is inadequate for the needs of the lactating female, and that this deficiency is reflected in the offspring.

Administration of active curative supplements.

To many of the offspring from these litters, effective supplements have been given; these experiments have been as yet only of a preliminary nature.

The following supplements when added to the basal diet or fed directly to the young offspring have had a curative effect.

(1) Substitution for the "Glaxo casein" in the basal diet of "light white casein" has resulted in complete recovery of 15 animals from three different litters, control animals still maintained on the basal Diet F eventually dying. The rats from these three litters had been weaned at 28 days and segregated into two groups, the two groups being placed on Diets E and F. These results are indicated in Figs. 1 and 2.

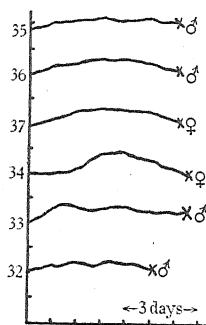


Fig. 1.

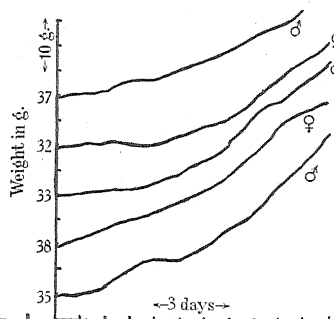


Fig. 2.

Fig. 1. Representative growth curves of control offspring maintained on Diet F. Weaned from parent 28 days old. x denotes death.

Fig. 2. Representative growth curves of offspring from the same litters, in which "light white casein" replaced "Glaxo casein" in Diet F at weaning.

(2) Extraction of the "light white casein" three times with cold 90 % acetone failed to remove the whole of the factor present in the protein. But the rate of recovery of the experimental animals when such extracted "casein" replaced the "Glaxo casein" in the diet, as indicated by gain in weight and disappearance of the sparse-looking fur, was much delayed.

(3) Administration of the 90 % acetone extract from "light white casein" resulted in two cases in the recovery of the young, control animals dying on being kept on the basal ration alone.

(4) Decidedly beneficial results have also been obtained by the feeding of a physin extract prepared from ox-liver. This extract represents material extracted from liver soluble in 90 % alcohol and 90 % acetone and was prepared as follows. Fresh ox-liver was minced and acidified to a p_H of 5.0, and the cells were broken down by means of rapid digestion by papain. After digestion an aqueous extract was made. Alcohol was added to a concentration of 90 %, the filtrate concentrated *in vacuo* at 40° and the residue dissolved in water. Acetone was then added to a concentration of 90 %. This precipitates further material. Approximately 380 mg. of acetone-soluble material are obtained from 100 g. fresh weight of liver. This material was fed in doses equivalent to 1-2 g. of original liver. A decided improvement was apparent in the animals receiving

this supplement, the outward indication of which was a rapid recovery and gain in weight, and complete disappearance of all sparse-looking fur. Death followed in control animals not receiving the supplement (Fig. 3).

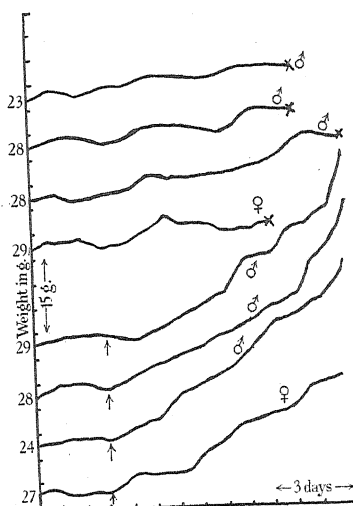


Fig. 3. Representative growth curves of offspring from litters bred on Diet F. Offspring separated from mother at 28 days. ↑ Supplement; physin extract from fresh ox-liver. × denotes death.

Modifications of the synthetic diet.

(a) *Manganese.* As Orent and McCollum [1931] have demonstrated the insufficiency of a diet completely deficient in inorganic manganese for the normal requirements during lactation, the content of inorganic manganese has been varied. Diet E' represents the composition of the original synthetic diet with

Table II.

	E'	E	B	F
Diet of mother from weaning	E'	E	B	F
Diet of offspring	E'	E	B	F
No. of litters on experimental diet	21	30	16	25
Litters of mothers showing immediate lactation failure at birth	11	13	10	14
Litters suckled at birth	10	17	6	11
Litters successfully weaned	10	17	6	None*
Total no. of rats born	155	224	124	189
Total no. of rats born whose mothers commenced feeding at birth	68	122	41	93
Total no. of rats alive at 14 days	66	120	40	87
Total no. of rats alive at 28 days	66	118	40	43
Total no. of rats alive at 5-6 weeks	65	118	40	10

* 2 partially successful.

an increase of inorganic manganese to approximately 0.01 % of the ration. No significant differences, however, were noted using this diet.

(b) *Wheat embryo*. The composition of Diets E and F reveals their large content of wheat embryo: this had been included to provide an adequate supply of the vitamin B complex. It was thought possible that such a synthetic diet might well possess too high a standard of vitamin E, leading to a disturbance of lactation, and might thus be the explanation of the large number of mothers who failed to commence feeding their offspring. Accordingly the wheat embryo has been reduced in Diet B to 2 %, just sufficient to ensure an adequate supply of vitamin E. The results of breeding experiments on this diet have, however, still revealed the immediate failure of lactation at birth, and in general no significant difference was apparent between Diets B and E. The general results recorded in this paper are given in Table II.

DISCUSSION.

Comparison with deficiencies previously noted.

The results recorded in this paper bring out in striking contrast the nutritional differences between "Glaxo casein" (caseinogen) and "light white casein B.D.H." (Na caseinate). Certain differences have been noted before.

Coward *et al.* [1929] demonstrated the possibility of the existence of a new dietary principle necessary for the continued growth of the rat. Their basal ration was, however, deficient in vitamin E, and this objection might be raised in connection with their work. They found their growth factor to be present in "light white casein" and absent from "Glaxo casein." They also demonstrated its presence in other natural foods, *e.g.* ox-liver, milk, wheat germ. They concluded that, although possessing many properties suggestive of vitamin E, it was not vitamin E on account of its different distribution and more unstable nature. The experiments recorded here indicate quite conclusively that the deficiency studied here is not due to any lack of vitamin E.

Palmer and Kennedy [1927, 1, 2] came to the conclusion, as a result of work on synthetic diets, that a further dietary principle existed which was not identical with vitamins A, B, C, D or E. They were unable to obtain normal growth on a synthetic diet of a highly purified caseinogen, butter-fat, agar-agar and an alcoholic extract of fat-free wheat embryo. Much better growth was obtained if a commercial caseinogen was used instead of highly purified caseinogen. The rats failed in growth and developed symptoms closely allied to those noted in the present work. They found that alcoholic extracts of wheat embryo stimulated the growth rate. The main objection to their work lies in the possibility of their diet being deficient in vitamin B₂, since their wheat embryo extract which formed the sole source of the vitamin B complex was obtained by use of high alcoholic concentrations, *e.g.* 85-95 %.

The observations of Palmer and Kennedy and of Coward *et al.*, however, indicated the possibility of the existence of a further dietary principle necessary for normal growth. The results recorded in the present work have indicated the inadequacy of a synthetic diet, complete according to established criteria, for normal lactation and growth of the offspring. This deficiency is not due to vitamin E. That the deficiency is not due to a lack of the vitamin B complex is shown by the fact that many of the active supplements effecting a cure are completely deficient in vitamins B₁ and B₂, *e.g.* "light white casein," or extracts from this. Moreover, a diet such as Diet B employed here, in which the con-

centration of the vitamin B complex is smaller than that of either Diets E or F, is still adequate for supplying the vitamin B requirements both for growth and lactation.

Inadequate protein.

The possibility had to be considered that the deficiency studied here might be due to some unsuitability of the "Glaxo casein" as a source of protein. It might be argued that the death of the animals described above may have been due (1) to some inherent deficiency in the "Glaxo casein" caused by its method of manufacture; (2) to some toxic body present in this protein which upsets the maternal metabolism and lactation and which may be passed to the offspring.

Experiments to throw further light on this question are being promoted, but the following considerations would seem to negative these views. Rats taken from the stock dietary and placed on Diet F grow equally well as rats placed on Diet E. Moreover, addition of liver or fresh milk to parent animals breeding on Diet F, prevents entirely any of the deficiency symptoms described above. Further, the growth rate of offspring from such litters is equal in magnitude to that obtained when liver supplements the "light white casein" synthetic Diet E. It is difficult to see how these results could be obtained if "Glaxo casein" suffered from some inadequacy as a protein.

It is conceivable that the Glaxo protein may contain small traces of a toxic constituent, due to its method of manufacture, which may be injurious to the very young animal, having no effect on an animal some weeks older. The evidence at present in hand is antagonistic to such a viewpoint. Until more positive evidence of such toxicity is available, it seems more reasonable to adopt the deficiency hypothesis outlined in this paper.

Possible identity with the Coward factor.

The further question arises whether this deficiency is caused by an absence of the Coward factor or physin, and whether these postulated dietary principles are not identical.

Physin, as has been shown in a former publication, causes an acceleration of growth in young rats maintained on a basal diet of the nature of the diet E used in the experiments now reported. Such a diet, it was believed, should be entirely adequate in the Coward factor. Dr Coward has, however, informed me (private communication) that she believes that 20 % wheat embryo in the diet might not be sufficient for the production of maximum growth in a ration otherwise deficient in this factor.

It is thus conceivable that the stimulation of growth obtained in the previous work by the addition to the basal ration of liver may have intensified the growth rate because of a more adequate supply of this substance.

It has been shown in this paper that an active extract containing physin is effective in curing the deficiency symptoms noted above. Preliminary stages in the fractionation of physin have revealed its solubility in such solvents as 90 % alcohol, 97 % alcohol, 90 % acetone. These compare well with the reported solubilities of the substance curing the deficiency reported by Coward *et al.* These results are suggestive, but a definite answer to this question must be left for future work.

The dietary cause of the immediate lactation failure reported in this and earlier communications has still to be cleared up. That it is not due to simple inorganic manganese deficiency seems clear. Whether it may be looked upon as an acute form of physin or Coward factor deficiency remains to be determined.

In further work on this problem the ability of the rat to store the growth-promoting principle must be taken into account. The evidence at present to hand suggests that this ability may be considerable. Recent work in progress indicates that young rats weaned from the stock dietary used in this laboratory will grow well up to weights approximating to 200 g. on diets deficient in this factor.

Of equal importance in this respect is the nature of the stock dietary from which the experimental animals are derived. The inability to reproduce identical results under apparently the same external conditions may well be traced to these causes.

SUMMARY.

1. The results of breeding rats on several different synthetic diets have been recorded.
2. A deficiency disease apparent in the suckling young of rats receiving "Glaxo casein" as their source of protein has been described. Failure of growth, loss of fur and eventually death are the typical symptoms observed.
3. Nutritional differences influencing the deficiency disease have been shown to exist between "Glaxo casein" and "light white casein B.D.H."
4. Active extracts containing both physin and the Coward factor have been shown to cure this deficiency.
5. The possible identity of physin with the Coward factor is discussed.

I wish to express my thanks to Sir F. G. Hopkins and Dr L. J. Harris for their kind interest and advice, and to Miss V. R. Leader for her able assistance with the animals.

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CXLI. THE TECHNIQUE OF GLASS ELECTRODE MEASUREMENTS.

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(Received June 28th, 1933.)

IN the course of an investigation into the value of the glass electrode as a means of making continuous records of the hydrogen ion concentration of circulating blood, it was found, in the preliminary experiments in which estimations were made on single samples, that there were several sources of serious error. The difficulties encountered in devising a satisfactory apparatus for measuring glass cell potentials have been overcome, and a suitable form of thermionic valve electrometer has been evolved [Platt and Winfield, 1933]. The present paper is concerned with technical details which must be carefully considered before accurate results can be obtained consistently.

DESCRIPTION OF THE APPARATUS.

The glass cell system consists of an electrode vessel carrying in a waxed cork the glass membrane and a 0.1N HCl-quinhydrone half-cell. The electrode vessel is provided with a tap A and is connected by a 3.5 M KCl bridge, as

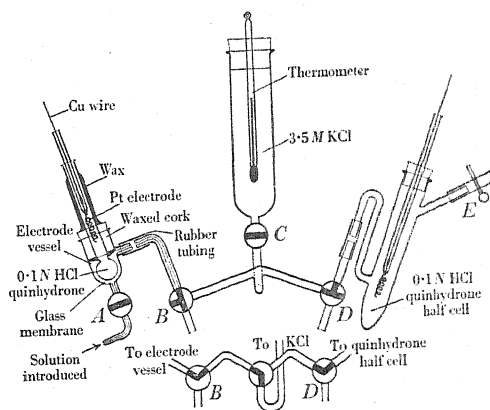


Fig. 1.

shown in Fig. 1, to another 0.1N HCl-quinhydrone half-cell. A reservoir containing 3.5 M KCl and fitted with a thermometer and a stopcock is so arranged that there are no differences in hydrostatic pressure throughout the system such as are likely to disturb the liquid-liquid junctions. The type of stopcock used in most of the experiments is shown in the figure, but a more convenient alternative

¹ Beit Memorial Research Fellow.

arrangement (see inset Fig. 1) is a set of 3 three-way taps, the limbs at 120° with V-shaped bores. These are substituted for *B* and *D*, the third interrupting the bridge so that the liquid-liquid junction may be made at *D* and preserved undisturbed in subsequent manipulations at *B*.

The procedure adopted was as follows. With tap *C* closed, tap *B* in the position shown and *D* in the corresponding position, the clip on the rubber tubing at *E* was opened and the bore of *D* flushed with 0.1N HCl-quinhydrone solution. Tap *C* was then opened, and the potassium chloride solution was washed through the bore of *D* as it was rotated in a clockwise direction for the formation of the liquid-liquid junction to the position shown in the diagram. The electrode vessel was filled through tap *A* from a Record syringe attached by narrow rubber tubing. The liquid-liquid junction was made in the same manner as at *D*, tap *C* being turned off when measurements were made. In this system it was found convenient, whilst making the junction at *B*, to maintain that at *D* by turning the latter through $\frac{1}{2}$ turn. The alteration of potential caused by this procedure was small—not greater than 0.5 mv.—and the original value was rapidly resumed.

The potential in the system was measured by the valve electrometer in conjunction with a small box type moving-coil galvanometer (Tinsley and Co.) and a slide-wire potentiometer (Cambridge Instrument Co.). Differences of 0.0001 volt were readily and rapidly detected using membranes of 20 to 200 megohms resistance. The electrometric system had a stable zero. The grid current flowing through the cell was of the order of 10^{-15} amp., giving a negligible fall of potential across the membrane. No appreciable current was taken from the cell, and the risk of polarisation of the membrane when the input circuit was out of balance was eliminated by means of a special switching device.

The glass cell system must be completely screened and the screen of this and the electrometer system earthed at a common point. The lead from the glass electrode was air-insulated as far as possible; for the rest, high grade heavily insulated flexible leads screened by flexible copper tubing were used. If the insulation of the cell is inadequate, the electrical leak may act as a shunt for the glass cell and lead to polarisation of the membrane [MacInnes and Belcher, 1931]. This is more liable to occur if the resistance of the membrane is high. The efficiency of screening and insulation was proved at intervals by determining the E.M.F. of a standard cell in series with a high resistance. A low reading was usually indicative of inadequate insulation, whilst defects in screening gave rise to irregular deflections of the galvanometer image when the standard cell and resistance were thrown into the input circuit.

DETAILS OF EXPERIMENTAL TECHNIQUE.

The selection, preparation and properties of the glass membrane.

The ability of a glass membrane to function as a perfect hydrogen electrode depends essentially on the chemical composition of the glass. There is general agreement that a clear soft soda-lime glass, free as far as possible from potash, alumina and borates, gives the best results [Hughes, 1928]. The stock used was supplied by the Corning Glass Co., New York (No. 015) and has the chemical composition— SiO_2 72 %, CaO 6 %, Na_2O 22 % by weight. According to MacInnes and Dole [1930], this glass has a low resistance and asymmetry potential and only a small error in alkaline solutions (0.1N NaOH).

The membrane may be a thin bulb in a simple or modified form; a thin membrane (about 0.001 mm.) at the end of a glass tube, as in the MacInnes and

Dole [1929] type, or a capillary may be used [Youden and Dobrotsky, 1931; Voegtlin and Kahler, 1932]. We have had considerable success with the simple bulb type which is easy to make and manipulate. The method followed is essentially that described by Voegtlin, de Eds and Kahler [1930], except that only a small portion of special glass is used. This is fused to a supporting tube of ordinary soft glass and is then drawn out to a capillary which is broken off about 1 cm. from the shoulder formed. The glass of the capillary is melted in a micro-burner into a centrally placed globule from which the bulb is blown. Prolonged manipulation in the flame should be avoided, as devitrification and annealing impair the properties of the glass for electrode purposes.

The supporting tube is not made of the glass 015, since, according to MacInnes and Dole [1930], 7×10^{-10} equivalent of base is given off from this glass per sq. mm. of surface in 24 hours. The composition of the glass used for the shank is usually such that its hydrogen electrode function is imperfect, so that it is desirable that there should be no appreciable current flow across it. Membranes of minimum thickness are therefore used in conjunction with relatively thick supporting tubes of low conductivity glass [MacInnes and Belcher, 1931]. Deviation currents, occurring with thick membranes and varying in amount according to the nature of the fluid on the two sides of the electrode and the area of the surfaces involved, are described by Kahler and de Eds [1931]. These authors suggest that a guard ring may be used for the electrode if the shank is short and wet. Reference has been made above to the danger of polarisation of the membrane due to leakage current, especially if thick membranes are in use. We have, however, had no trouble with the supporting tube when this is dried and painted with hot high melting-point paraffin wax.

A rough selection of the bulbs prepared by the foregoing method is made by filling them with water and taking those scarcely visible when inspected under water against a white background. Excluding occasional membranes containing minute holes due to rupture of air bubbles in the glass, those obtained by this method are found to be of suitable resistance and, with proper treatment, have low asymmetry potentials.

The value of the resistance of the membrane may be required, especially if the design of the potential measuring system is such that the cell resistance is a limiting factor. A method of measuring the D.C. resistance by applying only small potentials across the membrane may be used [MacInnes and Dole, 1930; Kahler and de Eds, 1931], or an approximate value may be obtained on a calibrated galvanometer when a known voltage (say 10 volts) is applied across the membrane when it is set up for taking a strain potential (see later). The value of the resistance of the membranes we prepared varied from 50 to 150 megohms and was generally higher than that usually obtained with the glass 015, since quite small bulbs (approximately 1 cm. diameter) were used. It is noteworthy that the values obtained by MacInnes and Belcher [1931] for the A.C. resistance of glass membranes are very much lower than, and roughly proportional to, the apparent D.C. resistance. This difference is, no doubt, largely due to a polarisation E.M.F. As a point of some practical importance it may be noted that the D.C. resistance of the glass membrane falls considerably with rise of temperature [Kahler and de Eds, 1931].

The glass membrane may be cleaned by steaming [Kerridge, 1925] or by soaking in dil. HCl followed by distilled water, preferably for several hours [Fosbinder and Schoonover, 1930]. The membrane should not be treated with strong alkalis or concentrated solutions of electrolytes. It should not be allowed to dry after preparation, since it depends for its hydrogen electrode function on

the water content of the glass phase [MacInnes and Belcher, 1931; Dole, 1931, 1932; Ssokolof and Passynsky, 1932]. We found that the membrane may be cleaned with alcohol and ether [Stadie *et al.*, 1931], though Dubois [1932] states that this procedure disturbs the stability of the cell he used. Contamination of the membrane with grease or paraffin should be sedulously avoided.

The terms zero correction, glass strain or asymmetry potential have been applied to the potential in the glass obtained when the solution is the same on both sides of the membrane. This entity is of importance, since, if large, it is likely to vary frequently and considerably. Even when using a suitable glass, large and variable asymmetry potentials may follow annealing, drying or chemical treatment. We have confirmed the findings of Greville and MacLagan [1931] that potentials may occur and persist when an unbalanced current flows in the input circuit of the electrometer. The glass potential has been shown to vary according to the p_H of the solution bathing the membrane [Kahler and de Eds, 1931].

Preparation of half-cells.

Different workers have used various combinations of calomel, silver-silver chloride and quinhydrone half-cells in conjunction with the glass electrode. We have used the following system:

Pt	0.1 N HCl- quinhydrone	Glass membrane	Unknown solution	3.5 M KCl	0.1 N HCl- quinhydrone	Pt
	p_{H_2}		A	a	b	
			p_{H_1}			

The platinum electrodes were made by fusing 2 to 3 in. of heavy gauge platinum wire to a piece of copper wire, then sealing the platinum into a piece of glass tubing of convenient size. It is easy to obtain a satisfactory seal if the hot electrode is covered with heated asbestos fibre and then allowed to cool slowly. The seal can be tested by heating the electrode in boiling water and allowing to cool, when the presence of a crack or of incomplete fusion is shown by the entrance of water into the glass tubing. The platinum was cleaned by heating up to 125° in chromic acid cleaning mixture, then slowly cooled, washed with tap-water, then with distilled water and kept in 0.1 N HCl till required [Morgan *et al.*, 1931]. 0.1 N HCl, kept at 38° was saturated with quinhydrone (B.D.H.) and the excess removed by filtration, nitrogen being bubbled through the solution at intervals. Büllmann and Jensen [1927] recommend 0.005 M quinhydrone solution (1.1 g. per 100 cc.), *i.e.* about one-third the amount required for saturation (3.5 g. per 100 cc. at 18°).

In comparison with calomel half-cells, the quinhydrone half-cells are easy to prepare and, using matched platinum electrodes, they can be reproduced to give potentials differing by less than 0.00001 volt [Büllmann and Jensen, 1927; Morgan *et al.*, 1931]. Compact forms, easily insulated, can be used. They do not exhibit the troublesome hysteresis phenomena with change in temperature found with calomel half-cells. Erratic values for quinhydrone half-cells may, however, be obtained at 38° if oxygen is not excluded. It is, moreover, convenient to have in the present system similarly constituted opposing half-cells; but, though these are matched initially, we find that a slow change in value takes place, possibly due to alkali from the glass in the smaller, or to oxidation in the larger, half-cell. If, however, the electrode is standardised at intervals against a buffer solution of known p_H , this change is not inconvenient. 0.1 N HCl is used in preference to a buffer solution, since Lammert and Morgan [1932] have shown

that quinhydrone electrode potentials are not nearly so accurately reproducible in solutions of other electrolytes as in 0.1 *N* HCl. It is an advantage too to have a solution of simple ionic composition in apposition to the membrane.

Liquid-liquid junction potentials.

An important consideration arising out of the use of 0.1 *N* HCl is that a significant junction potential is introduced in the cell chain at *b*. The values given for the potential at this junction vary, according to different authors, from 2 to 5 mv. Gross discrepancies are recorded in the junction potential, when such methods as saturated potassium chloride-agar bridges, narrow tubes or ground glass capped tubes are used in making the junctions [Maclagan, 1929]. There is a significant time change in the value of the junction potential which may vary considerably and inconsistently if these methods are adopted. Maclagan [1929] has shown that it is preferable to form the junction inside tubes not less than 3 mm. in diameter, thus conforming to the condition of cylindrical symmetry investigated by Guggenheim [1930]. Ferguson *et al.* [1932] claim to have reproduced in wide-bore taps a KCl-HCl junction to 0.1 mv., remaining constant over long periods to ± 0.04 mv. We have confirmed the finding of Maclagan, but find it inconvenient on account of difficulties with temperature control to use the method he recommends. The junctions were therefore made as described above in taps and tubes of internal diameter not less than 3 mm. In view of the time change of the junction potential at *b*, 5 to 10 mins. must be allowed for the attainment of a steady value, in order that accurate and reproducible results may be obtained. For the purposes of calculation it is assumed that the potential at *a* when the p_{H} of solution *A* is greater than 3 is always small and is the same within the limits of experimental error [Michaelis, 1926].

Standardisation of the cell system.

The discrepancies found in the values assigned to reference standards used in the measurement of p_{H} are such that, whilst small differences of p_{H} can be detected with considerable accuracy, absolute measurements cannot be established to less than 0.01 p_{H} [Clark, 1928]. The method we have used for standardisation of the glass electrode is based on the following considerations. If the membrane functions as a hydrogen electrode within the range of p_{H} covered in our experiments, then the potential difference (E_m) between the two sides of the membrane, when solutions of p_{H_1} and p_{H_2} are on either side, is given by

$$E_m = \frac{RT}{F} (p_{\text{H}_1} - p_{\text{H}_2}) \quad \dots\dots(1),$$

where R is the gas constant, T the absolute temperature, and F the faraday. The total E.M.F. (E) of the cell chain represented above is the algebraic sum of E_m , the glass potential (E_g), the liquid-liquid junction potentials at *a* and *b* and the difference, if any, between the potentials of the two half-cells. If the sum of the latter three factors be represented by e , then

$$E = E_m \pm e = \frac{RT}{F} (p_{\text{H}_1} - p_{\text{H}_2}) \pm e \quad \dots\dots(2),$$

p_{H_2} is constant and is taken as 1.08 [Clark, 1928]. p_{H_1} varies according to the solution in the electrode vessel.

Put

$$E_c = \frac{RT}{F} p_{H_2} \pm e \quad \dots\dots(3).$$

E_c is referred to as the cell constant; substituting in equation (2)

$$E = \frac{RT}{F} p_{H_1} - E_c \quad \dots\dots(4).$$

It is desirable to determine, firstly whether the membrane does in fact function according to the relationship between E_m and p_H expressed in equation (1) and, secondly, whether the other potential differences contributing to the total E.M.F. of the system remain constant. These conditions are fulfilled if identical values for the cell constant are obtained, using a series of phosphate buffer solutions of known p_H value. The buffer solutions were carefully prepared according to the method of Hastings and Sendroy [1924]. They were kept in the refrigerator in waxed bottles protected from contamination by CO_2 . We found that the differences of p_H as measured by the glass electrode agreed within the limits of experimental error with the values given by Hastings and Sendroy for the p_H of these buffers. When the value of E_c has been determined, the value, p_{H_x} , of an unknown solution introduced into the electrode vessel is easily found, for, if the potential of the system now is E_x , then

$$p_{H_x} = \frac{E_x + E_c}{\frac{RT}{F}} \quad \dots\dots(5).$$

The value of E_c is best determined by using buffer solutions of reaction and composition similar to those of the unknown solution. The cell system should be standardised carefully at frequent intervals, since we find, as Stadie *et al.* [1931] also have observed, that for various reasons E_c may slowly alter.

When 0.1N HCl is put into the electrode vessel in place of solution A, then $E_m = 0$. The E.M.F. measured when the second quinhydrone half-cell is connected directly to this solution without the interposition of the KCl bridge is the glass potential E_g , assuming that the two half-cells balance out exactly. If now the KCl bridge is introduced, the behaviour of the liquid-liquid junction may be studied. When the junctions are made simultaneously and in a similar manner they should give equal and opposing potentials, the E.M.F. of the system being again a measure of the strain potential. The time change of the junction can be investigated by remaking one junction after the other has had time to attain a steady value. Junctions made as described above show by this method a change of less than 0.5 mv., which was usually complete within 5 minutes. Because of the doubt as to the absolute value of the 0.1N HCl-3.5 M KCl junction, it is impracticable in making accurate measurements to compute the value of e and therefore to obtain a value for p_{H_x} from the formulae

$$E_m = E_x \pm e = \frac{RT}{F} (p_{H_x} - 1.08).$$

The system must therefore be standardised as described above.

The effect of temperature on glass electrode potential measurements.

As a temperature control of the glass cell system, various workers have used some form of air-bath; others, Fosbinder and Schonover [1930], Stadie *et al.* [1931] and Dubois [1932], have used some form of water-bath around part of the system. A copper water-jacketed air-bath, maintained at 38°, was used in our early experiments. Subsequently, on account of temperature fluctuations on opening the door of the thermostat, this was kept in a constant temperature

room at 35–36°. Appreciable temperature variations still occurred during manipulations, giving rise to unreliable potential measurements. Better results have been obtained by using a large completely screened cabinet (3 ft. × 4 ft. 6 in. × 6 ft.) built of zinc-covered plywood on a welded iron frame. The cabinet was electrically heated by screened elements controlled by a long tubular mercury-toluene regulator and a relay, the air being mixed by a fan driven by a motor fixed outside the cabinet. Operations were carried out inside the cabinet, *e.g.* the removal of blood from an arm thrust through a heat-insulated opening in the wall, or the cell system could be manipulated from the outside through two felted arm-holes. Suitable arrangements were made for lighting, transit, ventilation, *etc.* The electrical recording apparatus was set up outside the cabinet and was kept at room temperature. Apart from temporary disturbances due, for example, to entering the cabinet, it was found that the air temperature could be maintained at $38^\circ \pm 0.2^\circ$, owing, no doubt, to the large capacity and the presence of a complete metal lining. The temperature of the potassium chloride solution in the reservoir under these conditions was $37^\circ \pm 0.1^\circ$.

Solutions kept in the air-bath were always at a lower temperature than 38°. It was imperative therefore to avoid surfaces from which cooling might occur. Even when the closed glass cell system described above was used, we found that potential equilibrium was only slowly attained (15 to 20 mins.). The initial reading for the p_H of a phosphate buffer solution introduced into the electrode vessel from a stock kept in the air-bath in a closed vessel gave a value about 0.02 p_H more acid than that ultimately observed when the system had come to equilibrium, all manipulations being carried out inside the air-bath. Only when the solution before introduction was slightly warmer than 38° were steady potential readings obtained immediately. When the temperature was raised still further to, say 40°, an apparent acid change was observed.

Temperature measurements have been made of solutions inside the glass cell and the electrode vessel by means of small rapid reading thermometers graduated in 0.1° from 34 to 42°. In spite of a constant air-bath temperature of 38°, the fluid inside the glass cell in one of these experiments eventually remained steady at 36.8°. Fluids put into the electrode vessel from a stock at 40° gave an initial reading of the temperature of the fluid in the glass cell of 37.1°, falling to 36.8° in about 15 mins. Evidence was obtained that a fall in temperature occurred in the course of transference to the electrode vessel. The time taken for temperature equilibration was about that required for the attainment of potential equilibrium in previous experiments under comparable conditions. The apparent acid or alkaline change obtained when, from the E.M.F. measurements, values for the hydrogen ion concentration were calculated, assuming a uniform temperature of 37°, was therefore shown to be due to the temperature anomalies. The size of these changes for given solutions can be calculated by substitution in the formula $E = \frac{RT}{F} (p_{H_1} - p_{H_2})$. Suppose $p_{H_2} = 1.08$ and p_{H_1} of a phosphate buffer solution is 7; at 37° then, allowing for a fall of 0.029 p_H in the phosphate solution for 18° rise [Hastings and Sendroy, 1924],

$$E_{37^\circ} - E_{36^\circ} = + 1.2 \text{ mv. } (\equiv 0.02 p_H).$$

Irregular temperature changes may follow fluctuations in the air-bath temperature. The components of the system are affected unevenly on account of differences in volume of the fluids concerned and the surface area of the containing vessels. The total E.M.F. is altered, since, in addition to the factor discussed above, the E.M.F. of the half-cells may differ by 0.00074 volt for

1° difference of temperature [Bilmann and Jensen, 1927]. The time lag of these changes in the case of both fall and subsequent rise was found to be considerable. The temperature and therefore the E.M.F. of the half-cell may be directly affected by the solution in the electrode vessel if the fluid immediately surrounding the platinum electrode is involved. Repeated washing of the membrane can be shown to produce potential variations in the same way. Dubois [1932], however, attributes the potential changes after washing to an electrical charge on the membrane caused by friction and dielectric absorption. The effect of temperature variations on liquid-liquid junction potentials is unlikely to be of practical significance in view of the results of Prideaux [1928]. Experiments have been made proving that the drifts of potential we described were not due to the time change of liquid-liquid junction potentials.

The foregoing considerations are of importance in relation to the so-called "acid change" in freshly shed blood described by Havard and Kerridge [1929]. Further details of this work on the measurement of the p_H of blood will be presented when a method now under investigation for making rapid and accurate measurements on small quantities of fluid is fully developed. It may be stated here without further comment that the results of Havard and Kerridge can be explained on the grounds of inadequate temperature control and that we have been unable to obtain any evidence of an "acid change" in blood of the type obtained in their experiments.

SUMMARY.

A detailed description is given of the design and construction of a glass electrode system.

Several possible sources of error are described and discussed, and the conditions necessary for obtaining consistent and accurate results have been investigated.

Careful temperature control is shown to be of considerable importance. Measurements made of the hydrogen ion concentration of buffer solutions may show apparent acid and alkaline changes due to temperature variations. Reference is made to the importance of these factors in the interpretation of the so-called "acid change" in blood.

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CXLII. DETERMINATION OF IODINE IN BLOOD.

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(Received May 30th, 1933.)

THE purpose of this investigation was to establish a reliable and accurate method for the quantitative determination of small amounts of iodine. The following procedure has been elaborated.

A weighed amount of normal whole blood, up to 10 cc., is placed in a nickel crucible of 50 cc. capacity, together with 2 g. of potassium carbonate (Merck's Blue Label). The crucible is placed directly in an electric muffle furnace heating to 500° in 30 minutes. Then the oven is adjusted to maintain this temperature, as indicated by a thermometer. Heating is continued for 4 hours. The charred mass is broken up and finely ground with a spatula, care being taken to retain every particle in the crucible. Depending on the amount of ash present, water is added to form a pasty mass such as will fall away from the side of the crucible. This paste is extracted with 95 % alcohol 4 times, using 7 cc. each time. The extracts are passed through a filter paper (Whatman No. 5, 7 cm.), the filtrate being caught in a round-bottomed pyrex dish of 30 cc. capacity. This alcoholic solution is evaporated to dryness on an electric hot plate layered with asbestos, boiling being carefully avoided. The dry residue is now taken up with 1 cc. of water and transferred to a tube of diameter 1 cm. and length 2.5 cm., in which are placed 10 or 12 small capillary tubes sealed at the upper end. The solution is made slightly acid with 0.1 N H_2SO_4 , a micro-drop of a saturated aqueous solution of methyl orange serving as an indicator. In order to oxidise the potassium iodide to iodate, 4 drops of a freshly prepared aqueous solution of bromine are added. The tubes are placed directly in contact with an electric hot plate. The capillary tubes ensure regular and steady boiling. The solution is boiled for 1 minute. 20 seconds after the initiation of boiling, the yellow colour of the bromine disappears. The tubes are immediately placed on ice. A small drop (0.02 cc.) of a potassium iodide solution containing approximately 100 γ of iodine is added. Iodine is liberated to 6 times the amount originally present. One drop of starch solution is added and the solution titrated to colourless using 0.01 N sodium thiosulphate in a micro-burette. (150 divisions of thiosulphate from the micro-burette used were equivalent to 1.08 γ of original iodine.)

It has been found, using the above method, that blood of rabbits under standard laboratory conditions contains 8.00 γ of iodine per 100 g. Normal male human beings for this reason showed blood-iodine contents of 6.50 to 9.00 γ per 100 g.

The method is also applicable to the determination of the iodine content of urine and other body fluids.

The lack of unanimity of opinion on this subject indicates that a satisfactory method had not yet been established. Two factors are possibly involved: the limitations of the methods themselves, and the inability of one investigator to interpret accurately and repeat the prescribed technical procedure of another. However, it is hoped that this simplified technique, as detailed above, will permit repetition with satisfactory results.

DISCUSSION.

The ashing process may be employed either with an acid or alkaline medium. Using acidification, the ashing must be carried out in a closed system, which makes it very complicated, and for this reason acidification was not used in this investigation. Pfeiffer [1928] employed the acidification method, which was later modified by Glimm and Isenbruch [1929] and has more recently been used by Baumann and Metzger [1932].

Von Fellenberg [1923] used the alkaline method, but noted that ashing carried out under certain conditions resulted in a loss of iodine. The work of Höjer [1928] and of Jochmann [1928] is of considerable importance in that they demonstrated the necessity of carrying out the ashing at a constant temperature. Scheffer [1930] also showed that the volatility of iodine in the presence of organic material was increased in the absence of alkali. Obviously, the ashing temperature should not exceed the melting-point of potassium iodide, which for the pure substance is 680° . The above observations indicated that in ashing, two conditions should be fulfilled: (a) a constant ashing temperature not above 500° ; (b) the presence of excess alkali. As suggested by Scheffer [1930], reported by Allot, Dauphinee and Hurtle [1932] and confirmed in this study, organic matter containing iodine could be burnt in the presence of potassium carbonate without loss of iodine. For the first condition, an electric muffle furnace fitted with an adjustable rheostat was found satisfactory. Potassium carbonate was used as alkali.

Roman [1929] reported that the complete ashing of the blood was not necessary for the complete extraction of iodine. The iodine in the carbon-containing ash is capable of extraction with alcohol, but it needs to be emphasised that water must be present for the complete extraction of iodine. Scheffer [1930] and Turner [1930] illustrated this experimentally. In the present investigation, when absolute alcohol alone was used in extraction, a recovery of only 60 % was found, whereas with added water complete extraction was obtained. In evaporating the alcohol, boiling should be avoided, a point not universally recognised.

The final estimation of iodine may be carried out colorimetrically or titrimetrically. For the colorimetric method the iodine is changed into the elemental form and then mobilised in chloroform or carbon tetrachloride. Realising the limitations of the colorimetric procedure [Turner, 1930], it was considered desirable to develop the titrimetric method.

Chlorine [Veil and Sturm, 1925], potassium permanganate [Roman, 1929] and bromine [Leitch and Henderson, 1926] have been used as agents to oxidise the iodide to iodate. Any oxidising agent would be suitable, provided that the excess could be neutralised or driven off and thus not interfere with the later stages of the titrimetric procedure. A saturated aqueous solution of bromine was found most suitable since the excess could readily be removed by boiling. As recognised by Reith [1929], the bromine water, to be efficient, must be prepared freshly each day. The time of boiling must be carefully regulated, since excessive

boiling leads to a loss of iodine. As reported by Allot, Dauphinee and Hurlley [1932], boiling for longer than 2 minutes involves a loss of iodine.

It was found that a definite ratio must exist between the iodine of the iodate and the potassium iodide added in order to obtain a true starch blue colour. This ratio was found to be approximately 1 : 100. Variations from this ratio produced a purple-red coloured solution, the colour of which did not always disappear at the true end-point. This phenomenon has been described at some length by Reith [1929] and Allot, Dauphinee and Hurlley [1932]. On this basis, a knowledge of the approximate amount of iodine present in an unknown material assists in its accurate estimation.

The sharpness of the end-point increases with a corresponding decrease in the volume of the solution and with an increase in concentration of the thio-sulphate used in titration. To limit the error involved in the first instance, the final volume was arranged not to exceed 1.5 cc. To limit the second factor a micro-burette was made from a 300° thermometer, the bore of which was uniform throughout its length. The upper end of this burette was bent to form an inverted U, the end of which could be immersed in the solution to be titrated. The level of the thiosulphate in the burette was controlled by a mercury sac containing a screw on the lower end of the burette. 0.01 N sodium thiosulphate was used and prepared freshly each day from a 0.1 N stock solution. Each 10 divisions of the burette delivered a volume of 0.321 mm.³ A definite blue colour could be detected and could be accurately discharged with thiosulphate on 0.2γ of iodine. One drop of a 1 % starch in saturated sodium chloride solution served as an indicator. By these means the titration error was reduced to a minimum. Air, bubbling from a fine tip, was used for stirring, too much bubbling being avoided, since it was found to drive off iodine. The point at which the blue colour did not return in 15 minutes was considered to be the true end-point.

Blank determinations using 10 cc. of water in place of blood showed consistent values of 0.2γ iodine. 1γ of iodine added, as inorganic or organic iodine, could be recovered to 96 %. When similar amounts were added to whole blood of a known iodine content, they could be recovered to 94 %. Therefore, the error of the method was less than 10 %.

SUMMARY.

A modified technique has been devised for the quantitative determination of iodine, in quantities such as are present in blood.

This method has an error of less than 10 %.

Contamination must be carefully avoided in micro-estimations of iodine.

Strict adherence to an established technique is essential. The results secured, if not strictly quantitative, are at least comparable.

I wish to take this opportunity of expressing my gratitude to Dr E. J. King and Dr F. G. Banting for their constructive criticism and helpful suggestions during the course of this investigation.

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CXLI. THE USE OF SOME MICRO-ORGANISMS IN SUGAR ANALYSIS.

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BACTERIOLOGISTS have long used gas or acid production from a series of sugars for the identification of bacteria. Conversely, there are systems, or partial systems, of identification of sugars based on the fermentative action of different organisms [Castellani and Taylor, 1917; 1919; 1922; Kendall, 1923; Kendall and Yoshida, 1923].

From a qualitative standpoint such reactions may have been satisfactory; quantitatively, their application has not been always successful. Early criticisms of these methods have been made by MacLeod [1921] and Höst and Hatlehol [1920].

Two improvements have, however, rendered sugar estimation by living organisms less subject to error. Hiller, Linder and Van Slyke [1925] used a large amount (approximately 250 mg.) of baker's yeast to ferment the sugar in 1 cc. of blood, the optimum time of incubation necessary to produce the greatest diminution in reducing power being 20 mins. at 38°. Somogyi [1927] washed the yeast until free from reducing substances, prior to its use. Since then there have been many variations in the determination of "fermentable sugar" by yeast, but all depend on the employment of a large amount of washed yeast, a small amount of sugar and a short period of incubation. It is possible, however, that we may require sharper definitions of the volumes and nature of the interacting cells and fluid. Thus Schrumpf [1932] denies the possibility of the separation of glucose and galactose by yeast, and suggests that the non-fermentable portion of "blood-sugar" is a secondary product of the yeast on the glucose.

Raymond and Blanco [1928, 1] found that fructose, mannose and sucrose are partially removed from solution by baker's yeast under conditions favourable to the complete removal of glucose, whilst galactose, arabinose, xylose and dihydroxyacetone are untouched. Harding and Grant [1931] showed that if baker's yeast is acclimatised to ferment galactose, it can then be used to remove galactose from solution under conditions analogous to the estimation of glucose by the original yeast. *Saccharomyces marxianus* [Harding, Nicholson and Grant, 1932-33] which also ferments galactose, has been found useful as an analytical reagent for the same sugar; moreover, this yeast does not possess the power either to ferment or remove maltose.

These results make clear the existence of a connection between the fermentative action of a yeast, and the ability of the same yeast to act as an analytical reagent under specified conditions. The present paper shows that the principle is capable of further extension, and it seems within the bounds of possibility that a complete system of analysis of carbohydrate mixtures may ultimately

be devised. We have been able to analyse a mixture of glucose, fructose, galactose, maltose, lactose and sucrose within an error of 7 %, using the limited number of biological reagents as yet available. Such methods of analysis are particularly valuable when only small amounts of sugar are available.

In attempting to extend the use of biological reagents to the quantitative removal of sugars from dilute solutions in this manner, we have turned naturally to those yeasts and bacteria which have already proved useful in the qualitative detection of sugars. We thus have examined a series of *Monilia* species (kindly supplied to us by Sir Aldo Castellani) and a number of strains of *Proteus vulgaris*. From these we have selected a few which seem valuable from our point of view.

The use of pure strains of organisms becomes a necessity if precise and reproducible results are to be obtained. The experience of Harding and Grant with galactose-acclimatized baker's yeast, which shows a variable action towards maltose, and the even more variable action towards maltose of Fleischmann's baker's yeast, as shown in this paper, illustrates this necessity.

EXPERIMENTAL METHODS.

Sugars were bought as pure and twice recrystallised. Approximately 0.1 % aqueous stock solutions were made, and from these were prepared the various mixtures and dilute solutions used in the experiments. The reducing values before and after the action of the yeast under examination were determined on 2.0 cc. of the sugar solution + 2.0 cc. of a modified Shaffer-Somogyi copper reagent, heated for 10 mins. in a rapidly boiling water-bath [Harding and Selby, 1931; Harding and Downs, 1933]. As far as we know any sensitive copper or ferricyanide solution can be used.

The cultural conditions of the *Proteus vulgaris* and the *Monilia* species are given under separate headings. Those of *Saccharomyces marxianus* have already been published [Harding, Nicholson and Grant, 1932-33].

Fermentation and removal power.

To signify the loss in reducing power suffered by a sugar solution when treated with a large excess of a yeast for a short period of time, we have in this and previous papers used the term "removal." Hiller, Linder and Van Slyke used the term "fermentation." Benedict [1928] also selected this term, though so far no evidence has been presented that under the particular conditions of the reaction the processes usually designated as alcoholic or acid fermentation are actually responsible for the removal of the sugar. Raymond and Blanco [1928, 1] used the terms "adsorption" and [1928, 2] "disappearance," the latter term denoting our lack of precise knowledge of this phase of yeast action. The closeness of the connection, however, between fermentative ability and removal power can be seen by a comparison of the removal power of the organisms on a series of sugars, shown in Table I, and the gas or acid production as shown in Footnote to Table I.

The main lack of correlation between fermentation and removal power is with galactose. There is no gas or acid production by *M. pinoyi*, yet 100 % removal is effected. *P. vulgaris* is inert fermentatively, yet galactose suffers a small removal loss. *M. krusei*, which produces neither gas nor acid from galactose, and does not remove any galactose from solution when the sugar concentration is 20 mg./100 cc. or less, removes a small percentage when the sugar concentration is 50 mg./100 cc. This is not true of the action of *M. krusei* on maltose, lactose or arabinose. Both *S. marxianus* and *M. macedoninensis*

Table I. Showing percentage removal by some organisms of a series of sugars.

Organism	Washed wet- weight g.	Incu- bation time mins.	Gluc- ose	Fruct- ose	Mann- ose	Galact- ose	Malt- ose	Lact- ose	Arabin- ose	Xyl- ose	Sucr- ose	mg. sugar in 10 cc. solu- tion
<i>Proteus vulgaris</i>	0.75	30	100	0	0	21	0	0	0	0	0	1.5
<i>Monilia krusei</i>	0.5	30	100	99	100	0	0	0	0	—	0	2
<i>Saccharomyces marxianus</i>	0.5	30	100	100	100	100	0	0	0	0	100	2
<i>Monilia tropicalis</i>	0.5	20	100	100	100	28	100	0	0	0	5	2
<i>Monilia pinoyi</i>	0.5	30	100	93	90	69	100	0	0	—	0	2
"	0.75	30	100	99	100	80	100	0	0	—	0	2
"	1.0	30	100	100	100	100	100	0	0	—	0	2
<i>Monilia macedoninensis</i>	0.5	30	100	100	100	21	0	0	0	—	100	2

The removals have been carried out in neutral aqueous solution.

Footnote to Table I.

The gas or acid production was determined on a sterile 1 % solution of the pure sugar in peptone broth by the usual bacteriological technique.

Proteus vulgaris. Glucose: acid 12 hours, gas 72 hours. Fructose, mannose, galactose, maltose, lactose, arabinose, xylose, sucrose: no acid or gas in 72 hours.

M. krusei. Glucose, fructose, mannose: acid and gas 12 hours. Galactose, maltose, lactose, arabinose, xylose, sucrose: no acid or gas in 72 hours.

S. marxianus. Glucose, fructose, mannose, galactose, sucrose: acid and gas 12 hours. Maltose: acid in 48 hours, no gas in 72 hours. Lactose, arabinose, xylose: no acid or gas in 72 hours.

M. tropicalis. Glucose, fructose, mannose, maltose: acid and gas 12 hours. Sucrose: acid 12 hours, gas 72 hours. Galactose, lactose, arabinose, xylose: no acid or gas in 72 hours.

M. pinoyi. Glucose, fructose, mannose: acid and gas 12 hours. Maltose: acid 12 hours, gas 48 hours. Galactose, arabinose, xylose, sucrose: no acid or gas in 72 hours.

M. macedoninensis. Glucose, fructose, mannose, sucrose: acid and gas in 12 hours. Maltose: acid in 12 hours, no gas in 72 hours. Galactose, lactose, arabinose, xylose: no acid or gas in 72 hours.

slowly produce acid from maltose, but no gas in 72 hours, and neither shows removal power under our conditions. An alteration in properties is also to be noted in another section of the present paper where ordinary baker's yeast is found to have a removal action on maltose in a concentration of 20 mg./100 cc. but not at 10 mg./100 cc.

With the exceptions just noted, the removal power of a yeast, however, appears to be a function parallel with its rapid fermentative ability. Yet we hesitate to identify completely the two properties, quite apart from the fact that the galactose removals can be taken as evidence of the existence of an action other than fermentation. The removal of glucose can be extraordinarily rapid, so rapid indeed, that Somogyi [1927] at first considered the reaction as instantaneous. In all our experiments, 8 mins. has been sufficient to ensure the removal of 2-5 mg. of glucose by 250 mg. yeast at 38°. Fructose and mannose, which generally go through the same fermentative processes, require a much longer time to effect their complete removal, though by far the greater part of the removal is effected in the first few minutes. In this connection we would draw attention to the finding of Slater and Sand [1910] that the rate of entrance of glucose into the yeast cell is always more than sufficient (in presence of excess sugar) to maintain the maximum rate of fermentation. It seems to us a possibility that the sugar removal as shown under our analytical conditions may be associated with this rapid entrance into the yeast cell preliminary to the true fermentation process.

It is important to draw attention to the sharpness with which sugars can be separated by these methods. Not only is there a complete removal of the fermentable sugar, but there is no diffusion of the non-fermentable sugar in the yeast layer. Somogyi [1927] first noticed this, and it is illustrated by many

experimental figures in this paper. Van Slyke and Hawkins [1929] put this part of the technique severely to the test, when they incubated a 1:5 Folin-Wu blood-filtrate (previously treated with a small amount of yeast to remove the glucose) with an equal volume of centrifuged washed yeast, and found no alteration in the concentration of the residual non-fermentable substances. This, they found was not true of urine, though Harding and Selby [1931] and West and Peterson [1932] have been unable to confirm the removal of non-fermentable substances from urine under their own conditions.

The use of Proteus vulgaris as a reagent for glucose.

From the results of Table I it appears that *Proteus vulgaris* can be a valuable reagent for glucose. Our strain possesses neither fermentative ability nor removal power towards fructose, mannose and sucrose, and it would be in this field that it would prove most useful. The separation of glucose from sugars other than these, can be effected by many yeasts which are much more easily handled than this bacterium. However, the importance of glucose in the mammalian organism, and the value of a method for quantitatively separating this sugar from fructose and mannose, warrant the use of this bacterium as a sugar reagent, difficult as we have found the practical details of its application. Castellani and Taylor [1917; 1919; 1922] had previously reported *Monilia balcanica* as a specific gas-producer from glucose, but this organism is no longer available.

Strain. Of the three strains available from the American Type Culture Collection and three strains from the Department of Bacteriology of this University only one of the latter proved suitable. This particular strain was isolated 7 years ago by Prof. Holman. It has retained the same sugar-removal properties during the 2 years' work in this Department.

Culture. The bacteria are grown on 2 % neutral glucose-peptone broth for 24 hours at 38.0°. One loopful is transferred to a corner of 2 % neutral glucose-agar surface contained in a 26 oz. flat narrow-necked bottle and incubated for 24-36 hours at 38.0°. The growth is characteristic, spreading very rapidly from the inoculation corner over the whole agar surface. This habit of vigorous surface spread has assisted greatly in keeping a pure strain. The growth is washed off with 10 cc. distilled water for each bottle¹.

Separation. The bacteria can be separated from 1-2 cc. of liquid by the ordinary centrifuge at 3500-4000 r.p.m., but, with larger amounts of fluid there is always a considerable suspension, even after prolonged centrifuging. Pressure filtration through Seitz bacterial filter-pads, through Berkefeld or cellophane filters has proved too slow. The use of the Seitz pads, in addition gave products containing large amounts of reducing substances. The angle centrifuge of Lundgren at 3500 r.p.m. separated the bacterial suspension in 5 mins. Neither the ovoid glass centrifuge-tubes supplied by the manufacturer nor similar tubes made of pyrex glass proved satisfactory. Finally, thick aluminium tubes, made locally, were found serviceable. The lack of visibility inside these tubes was not found to be a hindrance.

Washing and yield. After the first centrifuging, the packed bacteria are stirred with 10 cc. distilled water and re-centrifuged. This is repeated 4 or 5 times. Usually the bacteria are then free from reducing substances. This method of washing is essential if, at the end of the action on the sugar solution, the bacteria are to be removed by centrifuging only. If, however, this latter removal is to be accomplished by filtration through kieselguhr it is sufficient to carry out only the first separation and one subsequent washing. From 1.0 to 1.5 g. of washed packed bacteria are obtained from each 26 oz. bottle.

Stability. The washed bacteria in 50 % suspension in water have shown variable stability. Some preparations kept at 4° have remained constant in their glucose-removal power for 4 days.

¹ The organism is kept in the same tube of broth culture for one week. Each week a loopful is transferred to an agar plate and re-transferred to broth after 24 hours. A stock culture is kept on an agar slant in a sealed test-tube and is transferred from agar to broth and back to agar once a month.

Other samples in the same length of time have altered, not only in glucose-removal power but in specificity. We have used the organisms within 12 hours of preparation.

Estimation of glucose. 1.5 cc. of 50 % suspension of the *Proteus* preparation are centrifuged in the aluminium tubes, the supernatant liquid poured off and adherent moisture removed from the tube by filter-paper. 10 cc. of the neutral sugar solution to be tested, containing not more than 1.5 mg. glucose are added, and the mixture stirred with a thin glass rod at 38° for 30 mins. The solution should now be at p_H 6.0-6.5 due to acid formation. 0.1 g. solid KH_2PO_4 is added if this is not attained. (In acid solution the bacteria flocculate rapidly and are more readily removed by centrifuging.) The greater part of the bacteria is then removed by a short period in the angle centrifuge. The removal of the last few cells must be accomplished separately by one of three methods. (a) By use of the thin high efficiency centrifuge-tubes supplied with the high angle centrifuge by the manufacturer. These tubes, if protected by rubber coverings, are satisfactory. The tubes hold only 5-7 cc. however, and in consequence a considerable time may be spent in this second centrifuging, if some 40-60 cc. liquid must be handled. (b) By filtration through a Berkefeld candle. This takes about 2 hours for 40-60 cc. liquid, but requires no attention. (c) By filtration through a layer of coarse kieselguhr on a small Büchner filter-plate. The kieselguhr is previously washed with HCl. Filtration is rapid, and if a considerable volume of liquid is available, this offers the best method for the removal of bacteria. The filtrate is bacteria-free, as tested by cultures, and sugars pass quantitatively. It is not possible to dispense with the first centrifuging, for if too many bacteria are present, the layer of kieselguhr clogs.

If the centrifugate or filtrate from (a), (b) and (c) contains ammonium salts, they are removed at this stage [Harding and Downs, 1933]. The reducing value of the sugar solution before and after *Proteus* action¹ is determined, using the requisite water controls. The difference between total and residual sugar represents glucose, in absence of galactose.

Action on galactose. The maximum removal of galactose we have observed is 27 % of a 20 mg./100 cc. solution, using 0.75 g. bacteria on 10 cc. of the neutral aqueous sugar solution for 30 mins. Using the same volume of sugar solution, bacteria and time of incubation, as under the previous subsection, we can estimate glucose in presence of galactose, following the summary of directions in the final section of this paper.

Action on urea. Different strains of *Proteus vulgaris* have been found to possess hydrolytic action on urea, producing ammonium carbonate [Day *et al.*, 1930]. 0.75 g. of wet-weight washed bacteria of our strain hydrolyses 10 cc. of a 250 mg./100 cc. urea solution to approximately 50 %. Such amounts of ammonia have been shown to lead to low results in sugar estimations [Harding and Downs, 1933]. As the conditions of the urea solution simulate a 1 in 10 diluted urine it becomes imperative, either to remove the urea, or to remove the NH_3 formed by its decomposition, before attempting sugar determinations by means of *Proteus*.

Recently Larson [1932] has discussed the problem of urea and NH_3 removal in presence of sugars without arriving at a satisfactory conclusion. We examined the removal of urea from urines by the use of $HgSO_4$ followed by either Na_2CO_3 or $BaCO_3$ [West and Peterson, 1932]. Even a second treatment with the mercuric salt still left sufficient urea to act as a source of NH_3 . The use of urease followed by permutit required such large quantities of reagents as to render the process impracticable for our method of sugar determination. Permutit, after the action of *Proteus*, gave high sugar recoveries. The removal of NH_3 as $Mg(NH_4)PO_4$ by the addition of KH_2PO_4 followed by MgO was found satisfactory after *Proteus* [Harding and Downs, 1933].

Analysis of glucose, fructose and sucrose. This mixture of sugars is important, both commercially and biologically. Given sufficient material the determination of the reducing power, before and after hydrolysis, together with the rotation of the mixtures provide data for the analysis. Given material amounting to only a few mg. per 100 cc. analysis by the above methods becomes impossible. Using the data of Table I, we find that *Proteus vulgaris* removes glucose, and leaves fructose and sucrose; *Monilia krusei* removes fructose and leaves sucrose. The sucrose can then be hydrolysed by dilute HCl, the solution neutralised, and the total invert

¹ In sugar determinations following the use of *Proteus*, and using the reagent detailed by Harding and Downs, it is essential that the I liberated from the KI be allowed to stand in contact with the Cu_2O for at least 3 mins. with occasional agitation before proceeding with the thiosulphate titration.

sugar determined by *M. krusei*, or the glucose and fructose estimated separately by *Proteus vulgaris* followed by *M. krusei*.

The details of an analysis of a known mixture of glucose, fructose and sucrose are shown in Table II.

Table II. Showing the details of an analysis of glucose, fructose and sucrose.

Glucose	10 mg.	} 100 cc. {	$\begin{cases} =1.72 \\ =1.69 \\ =0.00 \end{cases}$	} =3.41* cc. 0.005 N I for 2.0 cc. solution	
Fructose	10 "				
Sucrose	10 "				
					cc. 0.005 N I for 2.0 cc. solution
(1) Total reduction found	3.41
(2) Reduction after <i>Proteus</i>	1.70
3.41 - 1.70 = 1.71 = 10 mg. glucose removed by <i>Proteus</i>					
(3) Reduction after <i>M. krusei</i>	0.01
1.70 - 0.01 = 1.69 = 10 mg. fructose removed by <i>M. krusei</i>					
(4) Add 0.01 cc. N HCl to residual from (3) after removal of <i>M. krusei</i> . Heat 30 mins. in boiling water-bath. Add 0.01 cc. N NaOH.					
Total reduction	1.83
(5) Reduction after <i>Proteus</i> action on (4)	0.91
1.83 - 0.91 = 0.92 = 5.3 mg. glucose removed by <i>Proteus</i>					
(6) Reduction after <i>M. krusei</i> on (5)	0.01
0.91 - 0.01 = 0.90 = 5.3 mg. fructose removed by <i>M. krusei</i>					
(7) Equimolecular mixture of glucose and fructose = 10 mg. sucrose in analysis mixture.					

The amounts of wet weight washed organism and the time of incubation are those of Table I.
* Data from Table I [Harding and Downs, 1933].

This analysis is successful in absence of mannose. While mannose does not usually occur in natural mixtures containing glucose, fructose and sucrose, its absence should be established in those of unknown origin. We have found, so far, no biological method for the separation of fructose and mannose. Incorrect results will also be obtained in presence of maltose, as this sugar is partially hydrolysed by the acid.

Under these circumstances either the amount of sucrose can be calculated from the fructose produced by hydrolysis, or the maltose can be removed by *M. pinoyi* previous to hydrolysis. We have obtained satisfactory results by either method. In the presence of galactose a full analysis must be conducted according to the scheme given in the final section.

Application of Proteus to urines. Fasting human urine is cleared with H_2SO_4 and Lloyd's reagent [Folin and Berglund, 1922; Hamilton, 1928; Harding and Selby, 1931] with a final dilution of 1:10. The NH_3 is removed by KH_2PO_4 and MgO , and the total reduction determined. 25 cc. of the Lloyd filtrate are now brought to p_H 7.0 by one or two drops of 25 % NaOH. 10.0 cc. of this are added to 0.75 g. wet weight washed *Proteus* in the aluminium centrifuge-tube and incubated 30 mins. at 38° with stirring by means of a thin glass rod. 0.5 g. KH_2PO_4 is then added, the main portion of the cells removed by the high angle centrifuge, and the remainder by filtration through kieselguhr. The NH_3 is now removed by the addition of 0.75 g. MgO with agitation for 30 mins. The solution is brought back to p_H 6.5 by H_2SO_4 , and the residual sugar determined. The difference between the two determinations gives glucose in the known absence of galactose. Table III shows the action of *Proteus* in removing added glucose and its inertness towards added fructose, mannose, maltose, arabinose and xylose. We have also used *Proteus vulgaris* on urines cleared by $HgSO_4$ - $BaCO_3$ [West and Peterson, 1932]. We have sometimes found a continued action of the *Proteus* urease after centrifuging and the KH_2PO_4 - MgO treatment. The urease is destroyed in acid solution by a few minutes' heating at 70-80°.

It will be noted that Table III shows apparent small amounts of glucose (a-c) as present in normal fasting urine, whereas Harding and Selby [1931] had been unable to find any fermentable sugar in most specimens.

Table III. *Showing the action of Proteus vulgaris on urine and on urine with added sugar after treatment with Lloyd's reagent.*

Added sugar	Reducing value of urine after NH_3 removal (a)	Reducing value of urine + sugar after NH_3 removal (b)	Value of added sugar in urine after NH_3 removal (b) - (a)	Reducing value of urine (a) after <i>Proteus</i> (c)	Urine-sugar (a) - (c)	Reducing value of urine + sugar (b) after <i>Proteus</i> (d)	Added sugar after after <i>Proteus</i> (d) - (c)	% removal of added sugar
Fructose	1.04	2.80	1.76	0.84	0.20	2.61	1.77	0
Arabinose	0.73	2.05	1.32	0.40	0.33	1.72	1.32	0
Lactose	0.65	1.29	0.64	0.49	0.16	1.13	0.64	0
Maltose	0.83	1.52	0.69	0.45	0.38	1.13	0.68	0
Galactose	0.90	2.11	1.26	0.42	0.48	1.62	1.20	4
Glucose	0.97	2.74	1.77	0.73	0.24	0.73	0	100
Xylose	0.89	2.17	1.28	0.48	0.41	1.74	1.26	0
Mannose	1.04	2.10	1.06	0.64	0.40	1.69	1.05	0

The above values are expressed as cc. of 0.005 N I for 2.0 cc. urine (1:10) filtrate. For mg. added sugar see Table I [Harding and Downs, 1933].

Application of Proteus to Folin-Wu blood-filtrates. The method given for the estimation of glucose in aqueous solution can be applied to Folin-Wu blood-filtrates. The values obtained with two samples of dog's blood and six normal human fasting bloods as shown in Table IV differ in

Table IV. *Comparison of sugar removal by ordinary yeast and by Proteus vulgaris from Folin-Wu blood-filtrates.*

Blood No.	Total reducing substances	Residual reducing substances after yeast	Residual reducing substances after <i>Proteus</i>	Glucose mg./100 cc.
Dog 1	1.98	0.45	0.44	88
" 2	1.61	0.27	0.28	77
Man 3	1.57	0.28	0.31	73
" 4	1.70	0.35	0.33	78
" 5	1.72	0.35	0.33	79
" 6	1.71	0.34	0.33	78
" 7	1.84	0.40	0.40	83
" 8	1.52	0.40	0.39	66

Figures represent cc. 0.005 N I for 2 cc. blood-filtrate.

Table V. *Separation of glucose and non-glucose sugars from fermented Folin-Wu blood-filtrates.*

Value for residual reduction after yeast	Value after added non-glucose sugar	Value after added non-glucose sugar + added glucose	Value after <i>Proteus</i>	Added non-glucose sugar
0.31	0.39	2.07	0.42	Fructose
0.34	0.40	2.21	0.41	Maltose
0.40	0.46	1.79	0.44	Mannose
0.42	0.65	2.37	0.66	Lactose
0.42	0.87	2.53	0.88	Arabinose

Figures represent cc. 0.005 N I for 2 cc. blood-filtrate.

no wise from those ordinarily accepted. If "fermentable sugar" is determined by ordinary washed baker's yeast, using 0.25 g. of wet weight yeast for 8 mins. at 38° for 10 cc. of the same Folin-Wu blood-filtrate, the values are identical with those obtained by the use of *Proteus*. Table V shows in a similar manner the recovery of glucose added to Folin-Wu blood-filtrates previously fermented by ordinary yeast, and the inertness of the reagent to small added amounts of fructose, mannose, maltose, lactose and arabinose. The closeness of the residual reduction figures after

Proteus and after baker's yeast supports the use of the latter as a reliable reagent for glucose in normal bloods. In nephritic bloods with a high amount of urea, the use of *Proteus* for the determination of glucose without removal of NH_3 would lead to serious error.

The use of Monilia tropicalis for the estimation of maltose.

Maltose can be identified by its rotation, reducing power and osazone. These also supply data for its determination. In presence of other sugars or reducing material, and in small quantities, its estimation becomes one of difficulty. Barfoed's solution estimates monosaccharides in presence of disaccharides [Legrand, 1921; Tauber and Kleiner, 1932-33]. If the only disaccharide present is maltose, the difference between the monosaccharide reduction and the total reduction gives a measure of the maltose, but such estimations are obviously limited in application. Archbold and Widdowson [1931], in studying the sugar changes during apple storage, have utilised the differing rates of oxidation by Hanes's [1929] ferricyanide solution, and by hypiodite, for the simultaneous estimation of maltose and glucose. Smith [1932] has made use of the increased reducing power on hydrolysis as a basis of estimation of the same two sugars.

We have found no yeast which possesses a fermentative action on maltose alone. In Table I, however, are listed four organisms without any action on maltose and two organisms capable of its quantitative removal. Either of these last two (*M. pinoyi* or *M. tropicalis*), could be used as an analytical reagent. Of the two, *M. tropicalis* is the more active. (0.5 g. of the centrifuged freshly grown and washed cells remove 2-3 mg. maltose from 10 cc. liquid in 8 mins. at 38°.) Its action is slower on fructose and mannose, requiring 20 mins. for complete removal of the same quantity. *M. pinoyi* requires 30 mins. incubation to remove the same amount of maltose and is not quantitative in its action on fructose and mannose under the same conditions. *M. tropicalis* is thus preferable, though *M. pinoyi* may be used to separate maltose and saccharose. If *M. tropicalis* is preceded by any one of the four organisms having no removal action on maltose (*Proteus vulgaris*, *M. krusei*, *M. macedoninensis*, or *S. marxianus*) other sugars may be partially or completely removed. The use of *S. marxianus* followed by *M. tropicalis* gives a method specific for the estimation of maltose.

Culture and preparation. Inoculate a 2% glucose-peptone broth, phosphate-buffered to pH 5.5, with the organism, and incubate for 24-48 hours at 38°. Place 5-7 cc. of the 24-hour broth culture on 2% neutral glucose-agar in a 26 oz. narrow-necked bottle, and grow for 24 hours at 38°. Wash off the growth with distilled water. Centrifuge at 1500-2500 r.p.m. Wash several times with water until the washings are free from reducing material. Yield, about 2 g. wet-weight of yeast per bottle. The organism is made to a 50% solution with water and is used within 12 hours. The culture is controlled by microscopical examination for bacterial contamination. Every 4-5 weeks a new culture is prepared from a single colony picked from an agar-plate growth. The new culture should be tested against a series of sugars.

Estimation of maltose. (a) The total reducing power of 2.0 cc. sugar solution is determined. The sugar solution should not contain an amount greater than the equivalent of 20 mg. glucose per 100 cc. [see Table I, Harding and Downs, 1933].

(b) If sugars other than maltose are known to be present 12.0 cc. of the solution are placed in a centrifuge-tube, containing 0.5 g. wet weight washed *Saccharomyces marxianus* [Harding, Nicholson and Grant, 1932-33]. Incubate for 30 mins. at 38° with stirring. The *S. marxianus* is removed by centrifuging, taking care to remove completely any stray cells. The reducing power of 2.0 cc. of the centrifugate is determined. A control tube for the presence of small amounts of reducing material in the yeast is made under similar conditions with water instead of the sugar solution. The reducing value is corrected. The difference $a-b$ represents the reducing action of glucose, fructose, mannose or galactose, either singly or in combination.

(c) The remainder of the centrifugate from (b) (5-6 cc.) is added to a centrifuge-tube containing 0.5 g. wet-weight *M. tropicalis*. This latter has previously been added from the fresh 50 % suspension, the excess water has been removed by centrifuging and the interior of the tube wiped dry with filter-paper. The *Monilia* and sugar solution are mixed with a thin glass rod, or by tapping, incubated for 20 mins. at 38° with occasional stirring and then separated by centrifuging. The reducing power of 2.0 cc. of the centrifugate is then determined. Care is taken that no stray cells remain, previous to the "sugar" determination. A control tube with water and *M. tropicalis* is made simultaneously. The difference b-c represents maltose.

Application to blood and urine. Folin-Wu blood-filtrates were deprived of glucose by ordinary yeast, and to them were added a series of sugars. The removal of the added sugars by *M. tropicalis* is the same as in water and shows the same accuracy. Filtrates from dog's blood also gave similar removal figures.

The action of *M. tropicalis* on urine, either alone or in presence of added sugar has been tested as in testing the action of *Proteus*, except that no removal of NH_3 after fermentation is required. *M. tropicalis* removes glucose, fructose, mannose and maltose quantitatively. The removal of galactose from urine appears to be greater than from water solution. There is no action on lactose, arabinose and xylose.

*Use of baker's and brewer's yeasts for the separation of
glucose and maltose.*

Raymond and Blanco [1928, 1] found no removal of maltose by baker's yeast. Our observations on ordinary baker's yeast have convinced us that its maltose removal power is very variable, especially if 0.5 g. wet weight yeast is used and the incubation period at 38° is extended to 30 mins. On 10 cc. of a 20 mg. per 100 cc. solution of maltose, the disappearance of the sugar ranged from 16 to 77 % under such conditions. On the other hand, using 0.25 g. baker's yeast and incubating for 8 mins. at the same temperature, the removal of maltose was sometimes nil. As Raymond and Blanco only incubated for 3 mins., it is easy to see how they might fail to observe the disappearance of maltose. How complete can be the separation of glucose and maltose by the use of a small amount of yeast, coupled with a short period of incubation, can be seen from Table VI. Within experimental error and from a range of 1.25 to 10 mg.

Table VI. *Showing the action of 0.25 g. washed baker's yeast on maltose
and glucose mixtures after incubation for 8 mins. at 38°.*

Sugars		cc. 0.005 N I* for 2 cc. sugar solution	
Maltose mg./100 cc.	Added glucose mg./100 cc.	Original reduction	Reduction after yeast
20	0	1.23	0.84
10	0	0.62	0.60
5	0	0.31	0.29
2.5	0	0.16	0.15
1.25	0	0.07	0.07
20	1	1.39	0.92
10	1	0.78	0.60
5	1	0.47	0.30
2.5	1	0.32	0.15
1.25	1	0.24	0.07
20	20	4.68	1.10
10	20	4.03	0.63
5	20	3.75	0.31
2.5	20	3.59	0.13
1.25	20	3.50	0.08

* The iodide-free Shaffer-Hartmann reagent described by Harding and Selby [1931] was used in this experiment.

per 100 cc. the ordinary yeast cake (Fleischmann), not only had no action on maltose, but completely removed added glucose in concentrations of 1-20 mg./100 cc. It is to be noted, however, that at a higher concentration of 20 mg./100 cc. the yeast removed considerable quantities of maltose. The highest removal of maltose noticed by us with baker's yeast followed the use of a special sample, packed in ice by the laboratories of the Standard Brands Inc., and sent direct by express.

Two other observations convinced us that the freshness of the yeast was an important factor in the production of a high maltose-removal power. Cultures of baker's yeast grown in glucose-peptone broth, followed by growth on glucose-agar removed the maltose quantitatively. Brewer's yeast obtained fresh from the vat, and used immediately, also accomplished a 100 % removal of maltose, but at the end of 3 days the removal power had dropped to 64 %. Our standard criterion of removal in these experiments was the loss of reducing power experienced by 10.0 cc. of a 20 mg./100 cc. maltose solution when incubated for 30 mins. at 38° with 0.5 g. of wet-weight washed yeast.

To obtain baker's yeast with a very low maltose removal power required a number of experiments. Although it was evident that on standing the yeast lost some of its action, simple slow autolysis at 4°, or at room temperature, never yielded a product sufficiently low in maltose-removal power to be dependable as an analytical reagent. The effect of added phenol, or of desiccation at 38°, was to lower the maltose-removal power more rapidly, but attempts to accelerate these effects led to the death of the cell. Finally the simple expedient of allowing the small package cakes of yeast, as bought, and still wrapped in the foil covering, to remain on the laboratory bench for 7 days proved successful. The process is probably one of autolysis combined with slow desiccation. The packages were turned over daily. Any discoloured portions were removed before testing the action on sugars. The results of such an experiment and the effect of the subsequent reculture on the maltose-removal power are shown in Table VII. In both samples

Table VII. *Effect of "ageing" of baker's yeast on sugar removals. Figures show percentage removal produced by 0.5 g. yeast on 2 mg. sugar in 10 cc. solution at 38°.*

Time of incubation in mins....	Yeast Sample I						Yeast Sample II								
	After 7 days*			Fresh culture			As bought			After 7 days			Fresh cultures		
	8	20	30	8	20	30	8	20	30	8	20	30	8	20	30
Glucose	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Fructose	72	95	100	67	96	100	80	95	100	73	99	100	83	—	100
Mannose	88	97	100	—	—	—	16	19	32	—	—	100	—	—	100
Maltose	4	—	6	91	100	100	64	97	100	95	98	100	62	86	100
Saccharose	79	96	100	—	—	—	—	—	—	—	—	—	94	96	100

* Removal power of original yeast cakes undetermined.

the maltose-removal power is very low at the end of 7 days. The use of such yeast preparations acting, in small amount, for a short period of time gives a negligible removal of maltose. On the other hand the preparations are still active towards glucose, fructose, mannose and sucrose. Too long an exposure results in the death of the cell. The length of time of "ageing" must evidently vary from laboratory to laboratory, depending on temperature, humidity etc.

The foregoing experiments point to procedures for the analysis of mixtures of glucose and maltose. 0.25 g. wet-weight washed baker's yeast prepared from the "aged" partially desiccated packages and incubated for 3-8 mins., at 38° completely removes 2 mg. glucose. 0.5 g. wet-weight washed active brewer's yeast or baker's yeast just removed from a laboratory fermentation incubated 30 mins. at 38° completely removes 2 mg. maltose.

In view of possible variations each preparation of glucose-removing and maltose-removing baker's yeast should be tested on known mixtures of the two sugars. The experiments also indicate

one of the sources of the discrepancies sometimes encountered between different laboratories in the use of yeast for the separation of fermentable and non-fermentable sugars. Also, the strains or mixtures of yeasts used in commercial yeast cakes vary and contribute to failure of duplication of results. They emphasise the necessity of pure cultures.

Use of Monilia krusei.

M. krusei is an important organism from the standpoint of sugar analysis. It is the only one of our series which has no removal action on galactose at 20 mg./100 cc. concentration and has proved quite reliable in the separation of mixtures of glucose, fructose and mannose from that sugar. We have already noted that from higher concentrations of galactose, a small fraction is removed. In a previous section we have illustrated its use in the analysis of mixtures of glucose, fructose and sucrose. The cultural conditions of *M. krusei* are the same as for *M. tropicalis*.

M. krusei has the same removal reaction on sugars in Folin-Wu blood-filtrates and urines cleared with Lloyd reagent as in water.

Analysis of mixtures of sugars.

From the foregoing results and those of a previous paper [Harding, Nicholson and Grant, 1932-33] it is possible to construct a system of sugar analysis. The use of *Proteus vulgaris*, *M. krusei*, *S. marxianus* and *M. tropicalis*, combined with acid hydrolysis at appropriate stages, enables one to analyse a mixture of glucose, fructose or mannose, galactose, sucrose, maltose and lactose. A summary of such an analysis shows the following steps:

- A. Estimate glucose, fructose, mannose by *M. krusei*.
- B. Estimate galactose on residual fluid from A by *S. marxianus*.
- C. (In absence of galactose) estimate glucose by *Proteus vulgaris*.
A-C = fructose-mannose.
- D. (In presence of galactose) estimate fructose-mannose on residual fluid from C by *M. krusei*.
A-D = glucose.
- E. Estimate sucrose on residual fluid from A or D by hydrolysis, followed by use of *Proteus vulgaris* and *M. krusei*. (In presence of galactose or maltose calculate the sucrose from the fructose value.)
- F. Estimate maltose by *S. marxianus* followed by *M. tropicalis*.
- G. Estimate lactose on fluid from F by hydrolysis. Calculate the lactose from the galactose value, assuming 72 % hydrolysis [Harding and Grant, 1931].

The estimations A, C, F are made on the original mixture.

The details of analysis of the six sugars, each present at a concentration of 10 mg./100 cc. have been given in the preliminary account of this work [Harding *et al.*, 1932]. The percentage recovery of the sugars is shown in Table VIII.

Table VIII. *Showing percentage recovery from mixtures of sugars, analysed by the biological reagents.*

	I	II
Glucose	97.6	95.4
Fructose	101.7	101.1
Galactose	96.7	100.8
Sucrose	103.0	103.0
Maltose	109.6	105.1
Lactose	98.8	106.2

All four biological reagents act in aqueous solution at p_H 6.5-7.0. The cultural conditions must be strictly followed, otherwise considerable variations in the removal power are encountered. The application to biological fluids must always be accompanied by a considerable number of control experiments, in which the efficacy and specificity of the organisms are demonstrated. The number of sugars examined, while adequate for our present purposes, is not exhaustive. Reducing substances other than sugars may be removed, or have their reducing properties diminished. The presence in biological fluids of substances considerably modifying or even radically altering, the removal action of the organisms must be considered. Our own experience with *Proteus* on urine, where its action on the urea forms sufficient NH_3 to interfere with the accuracy of the copper reagent, illustrates another possible error. Used carefully, and with due regard to possible error, we believe biological reagents, such as we have described, will be of value in the study of many phases of carbohydrate metabolism, both in animal and vegetable physiology. Used indiscriminately, and without due regard to their limitations, they will prove inferior to the classical but slower methods of chemical identification of the sugars.

The possibility of such errors is reduced, if some removal of non-sugar material preliminary to the sugar estimation is adopted. Or the sugars may be separated in some insoluble combination from the other biological material and then liberated in aqueous solution. Under both sets of circumstances the absence of more than mere traces of heavy metals must be ensured. We have used the organisms after treatment of biological material by the following:

- (1) Lead acetate, followed by potassium dihydrogen phosphate.
- (2) Potassium dihydrogen phosphate followed by solid magnesium oxide.
- (3) Mercuric sulphate and barium carbonate followed by hydrogen sulphide [West and Peterson, 1932].
- (4) Copper sulphate and lime, with liberation of the sugar by sulphuric acid and hydrogen sulphide.

SUMMARY.

A number of micro-organisms have been examined as possible analytical reagents for sugars.

A close relationship between sugar removal power and fermentation has been shown.

Four of the organisms examined appear useful as biological reagents for sugars.

A strain of *Proteus vulgaris* has been developed as an analytical reagent for glucose.

Proteus is without removal action on fructose, mannose, maltose, lactose, sucrose, arabinose and xylose, but is variable towards galactose.

Proteus can be applied to Folin-Wu blood-filtrates and to urines after treatment with H_2SO_4 and Lloyd's reagent and after treatment with $HgSO_4$ and $BaCO_3$.

Details of the analysis of mixtures of glucose, fructose and sucrose are given.

Monilia tropicalis is extremely active in removal of maltose.

A method for the estimation of maltose is given, depending on the use of *Saccharomyces marxianus*, followed by *Monilia tropicalis*.

Baker's and brewer's yeasts show variation in their removal power towards maltose, depending on the freshness of the organism.

A method using "aged" and "fresh" baker's yeast for the separation of glucose and maltose is suggested.

Monilia krusei is a useful sugar reagent, as it only removes glucose, fructose and mannose.

A system of carbohydrate analysis, embracing glucose, fructose-mannose, galactose, sucrose, maltose and lactose, is outlined, and the result of the analysis of such a mixture by the biological reagents is given.

Some precautions in the use of organisms as analytical reagents for sugars are suggested.

The thanks of the authors are due to Mr G. Hern and Mr C. E. Downs for technical assistance.

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CXLIV. A RAPID METHOD FOR OBTAINING PROTEIN-FREE ULTRAFILTRATES OF BLOOD AND PLASMA.

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(Received June 20th, 1933.)

SINCE Smith [1928, 1, 2], working in these laboratories, developed a rapid method of ultrafiltration of plasma, we have had occasion to use the method frequently and on a large scale.

The following is an account of modifications in the technique which we have found necessary, (1) to improve the rate and yield of filtration, (2) to give membranes reliable and simple in preparation.

The chief difficulties experienced in using fine grade collodion membranes are firstly that they are very slow, and secondly that after a short time the rate of filtration is markedly decreased owing to clogging of the filter pores.

We have aimed at obtaining a membrane which will hold back protein and will filter rapidly at low pressure differences. There are two disadvantages of high pressure filtration. (a) The filters clog much more quickly; (b) the structure of the membrane may be actually altered so that its pore size is changed. With these considerations in mind we work at a pressure difference of about 70 cm. mercury. We prefer using a negative pressure, as it is more convenient in use and more economical, any good water suction-pump giving adequate pressure difference.

Methods devised to relieve blocking of the membranes are many. Pierce [1927] used shaking, Smith [1928, 1, 2] used a rocking filter with beads on the membrane, Elford [1931] used alternation of pressure. For filtration of fluids such as plasma we believe that the method described here gives very much more satisfactory results than any of the above.

METHOD.

The apparatus (see Fig. 1) comprises one or more filtering units. Each unit consists of a filter porcelain funnel *AA'*, similar in construction to a Seitz filter except that the two parts are clamped together by means of two metal rings held with bolts and wing nuts *BB'*. Soft rubber rings are interposed between the supporting grid *C* and the funnel portion and between the membrane and the reservoir portion of the filter.

The top of the reservoir is closed with a circular wooden cover *D*, the centre of which is pierced by a hole in which is held a short length (1 in.) of glass tubing. This acts as bearing for a solid glass T piece *E*, the side-limbs of which are inserted into two rubber sponges, *FF'*, cut as follows. They have cylindrical shape about $1\frac{1}{2}$ cm. in diameter and long enough to reach to within $\frac{1}{2}$ cm. of

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the central spindle and the wall of the reservoir. They are pierced longitudinally by means of a cork borer small enough to cause a tight fit on the glass arm. The sponge should be soft and loose-textured.

The T piece is connected by means of rubber tubing to a vertical shaft *G* which is made to revolve at a rate of one revolution every 3 seconds. There should be ample latitude for vertical movement of the shaft, as it is difficult to set up the filter exactly normal to the T piece. Constant pressure of the sponges

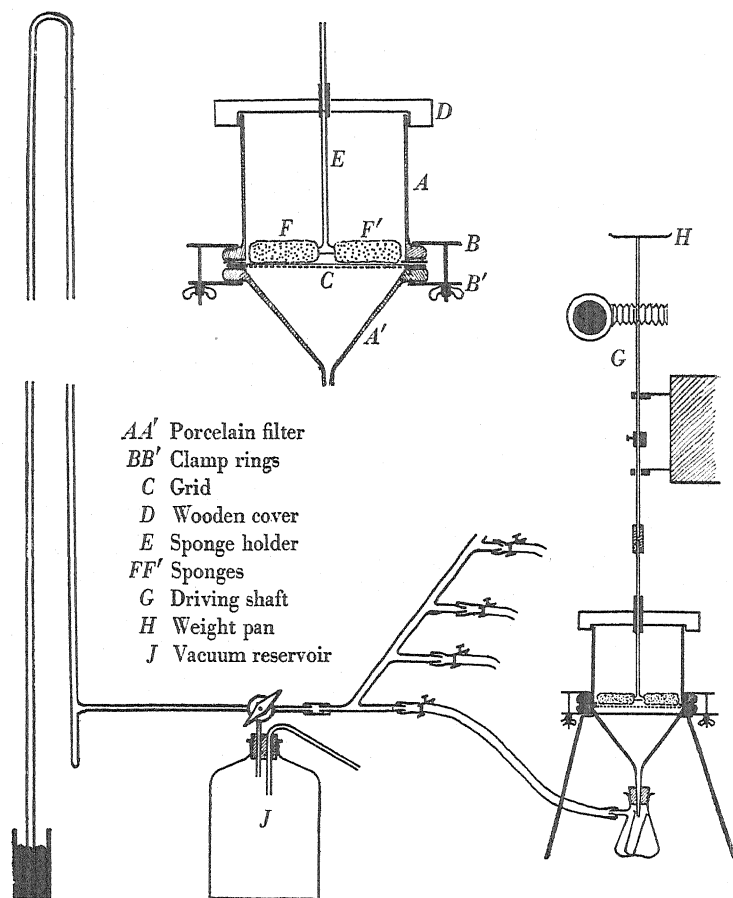


Fig. 1.

on the membrane is maintained by means of weights placed on the tray *H*. We have found that 50 g. is suitable for a 7 cm. filter, but the weight may need increasing when the residue becomes very viscous.

Suction is applied by the usual method from a good water-pump. A large reservoir is interposed between the pump and the filter battery in order to avoid extreme oscillations of pressure when connecting up a new filter while others are in use.

Several sponges are kept as spares. To clean them, they are first thoroughly rinsed in 3 % NaOH and then left for several days in 2 % Na_2CO_3 solution.

When subsequently rinsed with running tap-water and finally placed in distilled water for an hour or so they are ready for use. They should never be allowed to dry.

Preparation of the membranes.

Membranes were made with the following objects.

- (1) To keep back protein.
- (2) To have sufficient degree of surface hardness to avoid damage by the wipers.
- (3) To have sufficient strength to withstand pressures of one atmosphere.
- (4) To have a rapid filtration rate.

Two types of membranes have been found to fulfil these requirements.

(1) *Alcohol-ether-acetic acid-collodion membranes.* Pyroxylin is dried in a vacuum desiccator until of constant weight; this usually requires 3 to 4 days. From this a 6 % stock solution is made in a mixture containing 50 parts ether, 50 parts alcohol and 10 parts glacial acetic acid. After 3 days the stock can be used. We do not take particular precautions to render the ether and alcohol free from water. The ether used is Howard's anaesthetic ether and the alcohol is about 99.5 %.

The membranes are made from a solution containing 2 % pyroxylin by diluting down the stock with the mixture of alcohol, ether and acetic acid to the required strength.

The membranes are prepared on polished glass plates whose diameter is about 1 cm. greater than that of the filter. The plates are cleaned in dichromate and sulphuric acid, washed with distilled water, dried and polished with a silk cloth. The surface must be free from scratches and dust particles.

Holding the plate in the left hand about 5 cc. collodion are poured on to the centre, and by rapid tilting a thin film of collodion is formed covering the whole plate. By rapidly rotating in a vertical plane any excess of collodion is allowed to run off. The plate is now placed on mercury in a sulphuric acid desiccator. Here evaporation takes place in a still atmosphere—ensuring uniform drying of the membrane—from which moisture is precluded, thereby preventing condensation of moisture on the surface while the ether evaporates. In the absence of this latter precaution the film takes up moisture as it dries and a milky appearance or "blushing" of the membrane occurs. After a period of 5 minutes the plate is removed from the desiccator and the surface observed by reflected light. The appearance of a fine network pattern over the whole membrane surface indicates that drying is complete. The plate is now placed in distilled water. Gelling of the collodion occurs, the membrane contracts and floats off the glass in 1–2 minutes. It is then floated on to a filter-paper and placed in position in the filtering chamber, where it is washed free from acetic acid with distilled water.

These membranes are ideal for rapid filtration of plasma. They are, however, too fragile to be used repeatedly. In our experiments we found it desirable to filter plasma from patients under investigation and from control subjects through the same membrane, in order to eliminate the possibility of variable permeability of different membranes. We therefore attempted to prepare a stouter membrane impermeable to protein and suitable for successive filtrations.

(2) *Glacial acetic-collodion membranes.* The most suitable for our purpose were found to be glacial acetic-collodion membranes on a filter-paper basis similar to those described by Kreuger and Ritter [1930]. Hard Whatman (No. 52) filter-papers were immersed in collodion solution and the excess drained

away by rotation in a vertical plane. Gelling was brought about by immersion in distilled water and the excess acetic acid removed by washing in running water overnight.

Varying concentrations of pyroxylin in glacial acetic acid were tested. It was found that with concentrations of less than 4 % the membrane surface was damaged by the rotating sponges. Membranes of 4 % collodion can be used for several filtrations without deterioration of the surface. The rate of filtration under 70 cm. pressure is slightly slower than with the alcohol-ether-acetic acid-collodion membranes.

After filtration, the membrane surface is cleaned with 2 % sodium bicarbonate solution and the membrane washed on the filter with 50 cc. of the same solution followed by distilled water. It can then be preserved in distilled water until required for further use.

DISCUSSION.

We have obtained very satisfactory and consistent results using these types of membranes. Although the mode of preparation seems to lack that careful standardisation which many workers insist upon, we are convinced that the precautions taken are sufficient. Thus, for example, the exact volume of collodion used for each membrane is not measured, and one would expect varying rates of filtration from membrane to membrane owing to alteration in thickness. This does not seem to be the case, and we are of the opinion that the factor which determines the rate of filtration, and to a great extent the pore size, is the nature of the surface of the membrane. The thickness plays only a minor part. It is for this reason that the mode of drying the membrane is the most important part of the preparation to be controlled. The method is well suited for both purposes for which it was devised. For example, it is possible to obtain 25 cc. ultrafiltrate from 30 cc. plasma in 30 minutes. In certain injection experiments, we found that the time from taking the blood from the vein to the injection of it into the animal could be reduced to 45 minutes. As the whole filtration can be carried out at 0° and under sterile conditions, there is little danger of loss of labile substances through decomposition.

For large quantities it is equally suitable. The following figures give an idea of a typical filtration:

Vol. of plasma	1st hour cc.	2nd hour cc.	3rd hour cc.	4th hour cc.	5th hour cc.	6th hour cc.
450	100	85	70	60	50	25

SUMMARY.

1. An apparatus for rapid ultrafiltration and methods for preparing collodion membranes have been described with the object of removing proteins from blood, plasma and serum for the isolation of various constituents.
2. The method is suitable (i) for obtaining rapidly small quantities of ultrafiltrate for inoculation; (ii) for filtering large quantities.

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CXLV. THE ELEMENTARY COMPOSITION AND CALORIC VALUE OF THE FATTY ACIDS OF THE PHOSPHOLIPINS OF HUMAN SKELETAL MUSCLE.

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It is natural to assume that the fat laid down in the storage areas of the body is resistant to oxidation in virtue of its composition and saturation. As to the changes which occur in fat before being burned, chief consideration has been paid to the theory, originally suggested by Leathes, that the lipins may play some part in the desaturation and oxidation of the fatty acids. While it is generally held that the liver is the chief site of this desaturation, there appears to be little experimental proof of the rôle played by the lipins in the process. Kennaway and Leathes [1909] were themselves unable to find any gross difference between the degrees of unsaturation of the fatty acids of the triglycerides and lipins of the liver, such as to suggest a sharp differentiation into saturated and unsaturated.

The theory that the lipins are concerned in the mobilisation and transport of fat is also *sub judice*. Bloor [1924] considers that a large proportion of the unsaturated fatty acids of the blood are present as cholesteryl esters. Channon and Collinson [1929] have also noted that in the ox the phospholipin fatty acids of the blood have an iodine value varying from 71 to 90, whereas the fatty acids of the acetone-soluble fraction are much higher, varying from 121 to 143. These writers indicate that the relatively low iodine values of the blood-phospholipin fatty acids do not preclude the phospholipins from being considered as carriers of unsaturated acids from the liver to the tissues, as the fatty acids of these compounds are made up, according to Levene and his colleagues, of equimolecular proportions of saturated and unsaturated acids, and that the value of the unsaturated half of the mixed fatty acids, which have an iodine value of 80, will be 160. The latest available data [Boyd, 1933] indicate that in the human subject the degree of unsaturation of the fatty acids of the phospholipins of the blood is much higher than that of the total fatty acids—an observation at variance with the corresponding findings in lower animals.

The recent experiments of Sinclair [1932] also weigh against this general theory. Sinclair measured the rate of turnover of tissue phospholipins by determining the rate of change in the degree of unsaturation of the phospholipin fatty acids in the tissues of the rat following transfer from one distinctive diet to another. From his work he concludes that the phospholipins present in muscle, and presumably those in the other tissues as well, should no longer be regarded as intermediary products in the metabolism of fat.

The work of Mayer with Rathery and Schaeffer [1914-15] indicates that increased attention must be paid to the rôle of the phospholipins in cellular activity—particularly in the mitochondria. This is also the view of Bloor [1927].

In view, however, of the theories which assign to the phospholipins an active rôle in bringing about oxidation of fatty acids, it was thought necessary to obtain knowledge of the elementary composition and caloric value of the derived fatty acids of the phospholipins of an active metabolic tissue such as muscle and to determine if the heat liberated by their complete oxidation differed materially from the values found for the acetone-soluble fats [Cathcart and Cuthbertson, 1931].

ANALYTICAL METHODS.

The muscles selected in the first two cases corresponded to those chosen in our earlier paper, *viz.* the sartorius and rectus femoris.

Case I. 26 years, autopsy 9½ hours after accidental death. The body was that of a well nourished and apparently healthy man.

Case II. 31 years, autopsy 10 hours after death due to explosion. The body was that of a well nourished and apparently healthy man.

Case III. In this subject, aged 55 years, the gastrocnemius muscle of an amputated leg was extracted within 2 hours after the operation, which the patient's condition necessitated as the result of an accident.

The tissues were minced and stirred into twice their weight of acetone. After 2 days at room temperature the extracts were filtered through cotton cloth, and then the tissue was extracted three times with industrial alcohol and four times with ether. The first acetone extract was rejected. The extractions were carried out in dark brown bottles filled with CO₂. During the extractions the bottles were frequently shaken.

The filtered extracts were concentrated under reduced pressure at a temperature not exceeding 50°, a slow stream of CO₂ being passed through the fluid. When all the alcohol had been evaporated and a dark golden brown syrup remained, this concentrate was extracted with light petroleum, free from aromatic hydrocarbons, in a separator filled with CO₂, water being added to aid stratification. The aqueous layer was re-extracted. The combined extracts were evaporated to small bulk and then distributed in suitably sized centrifuge-tubes. Excess acetone was added to each, and after standing for some hours the tubes were centrifuged, the supernatant fluids decanted, the precipitates dissolved in the minimum of ether and the precipitation with acetone repeated. This process was carried out three times. The precipitated phospholipins were finally dissolved in ether.

Preparation of fatty acids. The ether extract was evaporated to dryness in a stream of CO₂ under reduced pressure. 50-100 cc. water and 2-5 g. NaOH were added according to the amount of material. After heating for 1 hour on a steam-bath under reflux, an equal volume of alcohol was added and saponification continued at boiling-point for another 3 hours. Water was then added, the cooled liquid twice shaken out with light petroleum in an atmosphere of N and the unsaponifiable matter rejected. The aqueous extract was then acidified with H₂SO₄ and completely extracted with light petroleum. This final extract after filtration was stored in a dark brown bottle until ready for analysis. It was found that this method was preferable to storage in the dry state.

The elementary analyses were performed by the writer personally using Pregl's technique. The iodine values were determined by Yasuda's [1931] micro-modification of the Rosenmund-Kuhnhenh method. This is more accurate

for lipin estimations than the earlier methods. A Darroch bomb was used for the caloric value determinations. This instrument allows duplicate estimations to be made within 0.01 %.

Scarcity of material prevented more complete tabulation, but as the data obtained agreed closely and as the supply of fresh human material was so uncertain, it was considered that the available data should be presented.

Case	C	H	O	Iodine value	R.Q.	Caloric value of 1 g.	Caloric value of 1 litre oxygen
1	76.10	11.84	12.06	111	0.712	—	—
2	75.80	12.20	12.00	—	0.705	9.496	4.73
3	76.20	11.98	11.99	111	0.711	9.516	4.76
Av.	76.03	11.98	11.99	111	0.709	9.506	4.745
Average value reduced to constant pressure*						9.521	4.75

* Cathcart and Cuthbertson [1931] *vide* Appendix B.

DISCUSSION.

Our earlier observations [Cathcart and Cuthbertson, 1931] on the acetone-soluble fats were made on the triglycerides and free fatty acids themselves, as it was believed that these molecules were burnt completely when used as fuel.

In considering the phospholipins, the derived fatty acids only have been analysed as it was believed that their fate was possibly different from that of the other constituents of the molecules.

The elementary composition, iodine value, respiratory quotient and caloric value and the other calculated data of these fatty acids of the phospholipins of skeletal muscle here described are of practically the same value as our corresponding observations on the triglycerides of the subcutaneous fat, but differ distinctly from the values obtained for the triglycerides of skeletal muscle—the latter containing more oxygen and less carbon.

If comparison be made between the elementary composition of these phospholipin fatty acids and the corresponding values of known higher fatty acids it will be found that the values C, 76.03 %; H, 11.98 %; O, 11.99 %; R.Q., 0.709 agree most closely with those of oleic acid, *viz.* C, 76.59 %; H, 12.06 %; O, 11.35 %; R.Q., 0.706. It is certain, however, that various unsaturated and saturated fatty acids go to make up this fatty acid complex [MacLean and MacLean, 1927].

Of the unsaturated group, recent work has shown that acids with 4 double bonds such as arachidonic acid occur in lecithin [Levene and Simms, 1922], and are widely distributed in the tissues and organs [Wesson, 1925]. Klenk and Schoenebeck [1931] have isolated from brain-lecithin an unsaturated acid containing 22 C atoms and 4 ethylenic linkages. The same acid has been found in liver-lecithin.

The saturated acids are mainly stearic and palmitic.

The iodine value of 111 which has been found for these fatty acids of the skeletal muscle-phospholipins, agrees fairly closely with the average value of 116 found by Bloor [1927] in his study of the fatty acids of the phospholipins of the jaw, diaphragm, neck and rump of the ox, particularly if account is taken that the method he used (Hanus's) tends to give values 2–4 % too high when applied to unsaturated fatty acids like linoleic [Yasuda, 1931].

While it may appear of questionable value to compare the caloric values of the triglycerides with those of the lipin fatty acids, no other course is available when the main consideration is the energy value and the significance of the respiratory quotient.

It is evident that the caloric value of a litre of oxygen utilised by the body will not be materially different if the material burned be the storage fat of the subcutaneous tissues or the fatty acids of the phospholipins of muscle. It will however be different if the material burned be the fatty acids of the phospholipins and not the triglyceride fat of muscle.

In our earlier paper we gave it as our considered opinion that the fats utilised for immediate combustion are the more highly oxygenated and probably more labile fats found in muscle and liver. There is at present no definite evidence to suggest the contrary.

SUMMARY.

1. Elementary analyses of the fatty acids derived from the phospholipins of the skeletal muscle of normal men gave an average of 76.03 % for carbon, 11.98 % for hydrogen, and 11.99 % for oxygen. The average respiratory quotient was 0.709; the average caloric value 9.521 cal. per g. at constant pressure, and the equivalent of 1 litre of oxygen at constant pressure was 4.75 cal. The average iodine value was 111.

My thanks are due to Prof. E. P. Cathcart for suggesting this investigation, and Prof. J. Shaw Dunn for supplying me with *post mortem* material. I am indebted to the Carnegie Trust for a grant in aid of this research.

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CXLVI. THE DEGREE OF UNSATURATION OF THE FATS OF HUMAN ADIPOSE TISSUE IN RELATION TO DEPTH FROM SKIN SURFACE.

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THE variations in the degree of unsaturation of the fats of adipose tissue in relation to depth from skin surface have received a considerable amount of attention in domestic animals, but no similar analyses of human material have been made.

Spaeth [1893] was one of the earliest observers to draw attention to the difference in iodine value and melting-point of fats obtained from the back and from the kidneys of fat hogs. The kidney fats proved to have lower iodine values and higher melting-points than the dorsal tissues. Mansfeld [Henriques and Hansen, 1901] had also noted that the skin-fats of pigs and also of cows had higher iodine values than the fats adjacent to viscera.

Lummert [1898] found that in three of the dogs which he examined there existed differences between the iodine values of the fats obtained from the skin and of those obtained from the region of the intestines from the same animal, but that there was no constant relationship to be found between the three different series as the average values revealed no real distinction.

On the basis of these observations Henriques and Hansen [1901] planned to determine if the above-mentioned differences in composition of fat from different parts of an animal were constant, and if the variations occurred to an even greater extent than had previously been described. Their method of extraction was simple, consisting in melting the fats by heat, a stream of CO₂ being blown over them to prevent oxidation. Von Hübl's method for the iodine value determinations was used. In addition they determined the solidification-points of the fats.

On the evidence of their experiments these workers put forward the interesting theory that the well-marked differences which they observed were due to the fact that storage fat is deposited in regions of different temperature, the most unsaturated fats being deposited in the coolest regions.

Since the present writers know of no similar data pertaining to human fat, it was thought of interest to present these analyses made in the course of a more exhaustive examination into the composition and distribution of fat in man.

ANALYTICAL METHODS.

The fats were obtained from comparatively fresh normal and obese human cadavers. The normals were all male subjects, the obese female.

The autopsies on the normals (fatal accidents) were performed from 9 to 17 hours after death, the average time being 12 hours. In the case of the fat subjects, the sections were performed from 3 to 25 hours after death—the average time being 13 hours. These latter cases included some of the most adipose female subjects which the writers have observed during a space of 4 years. In three instances in this group death was due to pulmonary embolism following trauma. In another case death was due to rupture of a cerebral aneurism and in the remaining two cases nephritis and carcinoma were the respective primary causes.

The fatty tissues analysed included the panniculus adiposus abdominalis, the omentum, mesentery, perinephric and epicardial tissues. In some cases the liver triglycerides were examined. The maximum thickness of the abdominal adipose tissues of the series (8.75 cm.) was observed in a female 160 kg. in weight. Preliminary experiments employing the extraction of the molten fat by heat were made but later abandoned owing to a tendency to contamination with tissue fluids. Subsequently the tissues were extracted three times with absolute alcohol and three times with ether in the cold. The extracts were concentrated to small volume by distillation under reduced pressure at 40° in a partial atmosphere of CO₂. The residue was extracted with light petroleum, free from aromatic hydrocarbons, the petroleum layer being separated, filtered and the solvent removed by distillation under reduced pressure at 40° in an atmosphere of CO₂. The fats were stored in an ice-chest until analysed, the last traces of solvent being removed just prior to analysis.

In the case of the liver the phosphatides were removed by repeated precipitation with acetone, traces of NaCl being present to facilitate separation.

After two preliminary observations on the iodine values of the derived fatty acids it was decided to consider the triglycerides and free fatty acids by themselves, as the removal of the small amount of unsaponifiable matter did not appreciably affect the relative significance of the data. It was also decided to abandon melting-point and solidification-point determinations, since the fats

Iodine values of fats derived from fatty tissues.

(a) Three normal subjects.

Tissue	Iodine value			No. of observations
	Maximum	Minimum	Average	
Panniculus adiposus abdominalis	73	67	70	3
Omentum	65	62	63.5	2
Perinephric	66	58	63	3
Epicardial	65	62	63.5	2
Liver (triglycerides)*	134	121	127	3

(b) Six obese subjects.

Panniculus adiposus abdominalis	Outer half layer	72	68	70	6
	Inner half layer	73	68	70	6
Omentum		71	67	69	5
Perinephric		65	61	63	3
Liver (triglycerides)		107	72	89	4

* [Cathcart and Cuthbertson, 1931.]

differed so little in their fluidity and since such determinations provided merely a qualitative test of the most solid and most fluid triglycerides and not a measure of the degree of unsaturation of the mixture.

The iodine values were determined by the method of Wijs. This method was used throughout to preserve continuity with the earlier specimens of the series which had been analysed by this technique. It is well known that Wijs's iodising solution is very reactive to cholesterol and it might be thought that the presence of this substance in varying amount might account for such divergence in the iodine values of the different fatty tissues as existed. Parallel observations by this method and that of Rosenmund and Kuhnhehn (Yasuda modification [1931]) on the fats and the fatty acids derived from them after removal of the unsaponifiable matter, indicated that the removal of the sterols, *etc.* did not alter the relative order and preponderance of the different fats one to another in respect of their unsaturation. The Rosenmund-Kuhnhehn method gave values about 6 % lower than Wijs's method.

An excess of Wijs's solution (about 200 %) was used.

DISCUSSION.

It was assumed that if any difference in the degree of unsaturation of the outer and inner tangential layers of the panniculus adiposus abdominalis did exist in the human subject, these obese cases would have exhibited the maximum effect considering the depth of this tissue.

It is apparent from the data that no real difference existed between these two layers in the six subjects examined, and further that the difference between the fat of this tissue and the omental fat was insignificant. The iodine number of the perinephric fat was, on the other hand, significantly lower.

In the case of the normal subjects, no division of the subcutaneous fatty tissue into outer and inner layers was possible. It is of interest that the degree of unsaturation was the same as that noted in the obese group.

On the other hand the omental fats had a lower degree of unsaturation than the subcutaneous. They were similar in value to the perinephric and epicardial fats, which in turn were of the same order of saturation as the perinephric fat of the obese group.

The close similarity in the degree of unsaturation of the subcutaneous and omental fats of the obese group may point to these tissues being storage areas for material of similar character.

It is apparent that the distinct differences in the composition of the storage fats at different depths from the skin surface which have been noted [Henriques and Hansen, 1901] in the lower animals, the dog excepted, are not, or at least only to a minor extent, reproduced in man. The general similarity of diet of these two omnivores may account for this, as the observations of Bhattacharya and Hilditch [1931] indicate that the more unsaturated the dietary fat, the less will be the differences in the unsaturation existing between the various body fats. This explanation is more probable than one based on temperature differences.

It is of interest that the triglycerides of the liver of the four obese subjects in whom this tissue was examined exhibited without exception low degrees of unsaturation as compared with the normal group, an observation in harmony with the morphological appearances.

SUMMARY.

1. The average iodine values of the fats of the panniculus adiposus abdominalis, omental, perinephric, epicardial and liver tissues of normal men were 70, 63.5, 63, 63.5 and 127 respectively.

2. In obese women the average iodine values for the fats of the outer and inner layers of the panniculus adiposus abdominalis, the omentum, and the perinephric and liver fats were 70, 70, 69, 63 and 89 respectively.

In conclusion we wish to express our thanks to Prof. J. Shaw Dunn for his kindness in supplying us with suitable material.

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CXLVII. INORGANIC CONSTITUENTS OF CEREBROSPINAL FLUID.

IV. THE POTASSIUM IN SERUM, SERUM-ULTRA- FILTRATE AND CEREBROSPINAL FLUID.

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(Received June 30th, 1933.)

A NUMBER of investigators have examined the cerebrospinal fluid for potassium and reported that it contains 25-50 % less than the serum. The averaged serum values of the different observers vary very much, and, although their averaged cerebrospinal fluid values are more consistent, the range of scatter of the individual cases differs considerably from one observer to another. Thus in serum Parhon and Werner [1932] found relatively little scatter (16.4-18.9 mg. per 100 cc.), but figures ranging from 15 to 27 mg. have been given by others. The lowest and most constant values (13.7-14.0 mg. per 100 cc.) have been reported by Dulière [1931], who separated the plasma from oxalated blood as rapidly as possible after removal from the body.

In the C.S.F. Dulière again found relatively constant potassium values, which averaged 11.17 mg. per 100 cc. Others, however, while agreeing on this average have found much wider variations, for example 10-18 mg. [Ballif and Gherscovici, 1932].

The literature on this subject has been reviewed by Nourse, Smith and Hartman [1925] and more recently by Lierle and Sage [1932] and need not be further discussed.

It is generally agreed that the whole of the potassium of serum is diffusible [e.g. Rona *et al.* 1924; Massaut, 1931, 1, 2], but Greene and Power [1931], who used *in vivo* dialysis in dogs, found 24 % non-diffusible.

If the whole of the potassium is in fact diffusible one would expect to find that any body fluid formed from the serum by simple physical means would contain about as much potassium as the serum. Thus, whatever may be true of the cerebrospinal fluid, this relationship might be expected in pleural effusions, hydroceles and ascitic fluids. Here again an investigation of the literature showed widely differing results. Loeb *et al.* [1922] obtained an average of 9.77 mg. K per 100 cc. in a series of fluids, mostly ascitic, whereas the corresponding serum value was 16.03 mg. per 100 cc. Three examples of potassium in oedema fluid given by Salvesen and Linder [1923] were 70-80 % of the serum values. The individual figures varied greatly and are higher than those given by Loeb *et al.* Greene *et al.* [1931], in experimental oedema in dogs, found that the potassium content of the fluid was the same as that of the serum. On the other hand ascitic fluid from human subjects had an average of 13 mg. K per 100 cc. compared with 17 mg. in their sera. Contrary to these findings are those of Dumitresco-Mante and Petrovano [1931] who found the potassium of centrifuged pleural fluid to be equal to, or (more generally) somewhat higher than, that of the corresponding serum.

Comparison has also been made between serum and ocular fluids, with equally divergent results. Tron [1926] using ox and horse, and Lebermann [1925] using rabbit, found only some 70 % of the serum-K in the vitreous and aqueous humours. Duke-Elder [1927], however, who has brought forward very complete evidence that the ocular fluids are true ultrafiltrates, obtained a close approximation between the serum- and aqueous-potassium. Stary and Winternitz [1932] also found close agreement between the potassium of the ventricle fluid of the eye and that of the serum-ultrafiltrate, both being somewhat higher than the serum value. Cohen *et al.* [1928] compared vitreous with cerebrospinal fluids and found average values of 24.9 mg. per 100 cc. for the former and 19.5 mg. for the latter. Both these figures are high and no serum values are given; nevertheless the ratio of the two suggests that they are not both formed from the serum in the same way.

In considering the reason for serum values being higher than those of the C.S.F. or other body fluids, one must remember the possibility that the higher serum values may be due to the potassium in the corpuscles leaking into the serum during the formation of the clot, since the lowest figures for serum potassium have been obtained on rapidly separated plasma [Dulière, 1931]. There is also the fact that some 24 % of the serum-potassium has been held by one group of workers to be non-diffusible [Greene and Power, 1931].

The present investigation has been prompted by these uncertainties and by the importance of the result in assessing how far the C.S.F. can be regarded as a serum-ultrafiltrate.

METHODS.

Blood was collected chiefly from in- and out-patients of King's College Hospital; some specimens were obtained from the National Hospital, Queen Square. The general technique has been described previously [McCance and Watchorn, 1931]. When for special purposes (see Table III) it was desired to separate the serum from the corpuscles as quickly as possible, the following procedure was adopted. A S.I.M.A. serum needle (Down Bros.) with rubber tubing attached was inserted into the vein. The patient was then bled with light constriction direct into a large paraffin-coated tube which was immediately centrifuged at high speed for 4-5 minutes. The supernatant fluid was pipetted off rapidly into another smaller centrifuge-tube and allowed to clot. This white clot was later broken up and clear serum obtained by centrifuging. The C.S.F. was withdrawn 15 minutes after the blood.

For the majority of the analyses Kramer and Tisdall's [1921] method was used. Determinations were made in duplicate at least, frequently in triplicate or quadruplicate. Some idea of the accuracy of the method may be obtained from the following figures obtained on the same large sample of mixed sera. Each ultrafiltrate figure represents a separate filtration.

Serum (mg. per 100 cc.)	Ultrafiltrate (mg. per 100 cc.)
26.40	29.04
27.09	27.59
26.40	27.22
26.08	29.74
26.71	27.59
—	29.24

A few of the later determinations (including all those in Table IV) were made by Jacobs and Hoffman's [1931] colorimetric modification of Kramer and Tisdall's method. This enables the precipitate of potassium cobaltinitrite to be

washed with alcohol, in which it is completely insoluble. This excludes also the possibility of high serum results being due to the oxidation of organic matter, *e.g.* protein debris, by the permanganate in the titration method. The colorimetric method gave closer agreement between duplicates than did the titrimetric and was of particular advantage in dealing with body fluids other than serum and C.S.F. in which a great deal of organic matter is sometimes precipitated. In these cases the coloured solutions were lightly centrifuged before matching in the colorimeter.

The method of ultrafiltration used was the same as that previously described [McCance and Watchorn, 1931].

RESULTS.

Table I shows the amount of potassium found in a series of sera, their corresponding ultrafiltrates and cerebrospinal fluids. The whole of the serum-potassium appears to be diffusible. The percentage shown in the ultrafiltrates

Table I. *Potassium in serum, serum-ultrafiltrate, and C.S.F.*

Diagnosis	Serum per 100 cc.	Ultra- filtrate	C.S.F.
Cerebral tumour	18.15	20.76	13.06
Taboparesis	17.63	19.06	12.93
Parenchymatous nephritis	20.24	21.55	11.23
Diabetes	18.54	20.24	—
Disseminated sclerosis	21.03	23.64	12.80
"	19.98	21.81	13.71
"	23.90	25.07	12.93
Neuro-syphilis	20.37	20.37	12.41
Cerebral tumour	23.05	24.14	10.82
Tabes	21.81	22.72	10.82
Amyotrophic lateral sclerosis	17.15	18.56	15.00
Cirrhosis of liver	18.56	18.69	—
? Syphilis	17.02	17.15	12.67
Cerebral tumour	17.66	19.58	10.75
Epilepsy	22.53	23.68	—
Meningo-vascular syphilis	17.56	19.27	12.67
Average	19.70	21.20	12.45

Table II. *Additional C.S.F.-potassium values.*

Diagnosis	C.S.F. (mg. per 100 cc.)
T.B. meningitis	13.19
"	11.49
"	13.45
"	14.07
"	11.62
"	12.95
"	12.22
Meningococcal meningitis	8.16
"	9.79
Chronic nephritis	11.78
Trauma with neurological signs	11.01
Spinal tumour	11.44
Tumour of cauda equina	7.96
Meningeal haemorrhage	12.69
Cerebral arterio-sclerosis	11.87
Cerebral cyst and tumour	11.58
Average	11.58

is actually higher than that in the serum because no correction has been made for the space occupied by the serum-proteins. Cerebrospinal fluid taken from the same patients at the same time invariably contained less potassium than the

serum. A further series of cerebrospinal fluids (Table II) also failed to give values approaching those usual for serum. All the sera in Table I had been left in contact with the corpuscular clot for some 6 to 18 hours before separation. A further series of comparisons therefore was undertaken in which the effect on the serum-K of leaving the serum in contact with the corpuscles was studied. From Table III it appears that there is no appreciable diffusion of potassium

Table III. *Potassium content of serum in contact with clot and of C.S.F.*

Serum (mg. per 100 cc.)	Time in contact with clot	Serum (mg. per 100 cc.)	Time in contact with clot	Increase (mg. per 100 cc.)	C.S.F. (mg. per 100 cc.)
18.31	0 hours	23.05	18 hours	4.74	10.82
17.66	0 "	20.22	18 "	2.56	—
15.78	0 "	17.09	10 "	1.31	11.44
18.23	0 "	21.08	18 "	2.85	13.37
17.87	0 "	18.27	20 "	0.40	15.15
18.27	0 "	21.15	19 "	2.88	13.26
15.39	0 "	17.58	18 "	2.19	12.69
17.15	0 "	17.15	5 "	Nil	—
18.51	0 "	18.82	7 "	0.26	—
17.02	0 "	17.28	7 "	0.26	—
19.70	0 "	19.91	7 "	0.21	11.97

out of the corpuscles for the first 7 hours after taking blood, but that later the serum-K slowly increases. These results therefore show that the potassium in the C.S.F. is generally much below that in the serum and that although potassium does tend to leak from the corpuscles after about 7 hours this does not account for the serum/C.S.F. relationship.

Table IV. *Potassium in various fluids.*

	Potassium		Protein	
	Serum (mg. per 100 cc.)	Fluid	Serum %	Fluid %
Hydrocele fluid	16.43	16.57	—	—
"	14.33	15.26	—	—
"	16.57	15.10	—	—
"	—	18.27	—	—
"	16.95	18.04	—	—
"	14.91	17.18	—	—
Pleural fluid	16.56	14.06	—	—
"	18.69	18.74	—	—
"	18.02	19.91	7.2	5.1
Ascitic fluid	19.30	17.96	—	—
"	17.44	16.08	2.7	0.18
"	13.32	16.28	—	—
Average	16.59	16.87		

Table IV shows that the amount of potassium in effusions was found to be approximately equal to the amount in the corresponding sera. This is the relationship characteristic of the ultrafiltrates and is in agreement with the findings of Dumitresco-Mante and Petrovino [1931].

DISCUSSION.

The average C.S.F.-potassium was 12.05 mg. per 100 cc., which is slightly higher than the figure usually given. Most of the values are close to this average, but the range of variation seems to be greater than in the case of calcium and

magnesium [McCance and Watchorn, 1931; 1932; Watchorn and McCance, 1932]. No constant variation of the cerebrospinal fluid-potassium in any one direction in any specific disease has been observed. The fall in magnesium in the C.S.F. in cases of meningitis is not accompanied by a fall in potassium. Further, Tables I and III show that no constant relationship has been found between the serum and the corresponding C.S.F.-potassium; high serum values were frequently accompanied by low C.S.F. values and *vice versa*. The average value for serum separated as rapidly as possible without the use of anticoagulant was 17.6 mg. per 100 cc., and the average for the corresponding cerebrospinal fluids was 12.7 mg. per 100 cc. In no case did we succeed in obtaining a serum value as low as that of the corresponding cerebrospinal fluid, nor such low values as Dulière [1931] reported. Even Dulière's sera figures were, however, higher than those of his cerebrospinal fluids.

Whatever may be true of sodium [see Dailey, 1931] it appears that, whereas the whole of the potassium of serum is ultrafiltrable (and the amount found in effusions can be thus explained), the level found in the C.S.F. represents at most 65-70 % of that found in the serum. Massaut [1931, 1, 2] and Kral *et al.* [1929], discussing similar results, consider that they are not in keeping with the view that cerebrospinal fluid represents an ultrafiltrate of the blood-plasma. With this opinion we are in complete accord and suggest that the C.S.F. should be compared with the intestinal secretions [Gilman and Cowgill, 1933] which are isotonic with blood but contain very different ionic concentrations.

CONCLUSIONS.

1. The whole of the potassium of serum was found to be ultrafiltrable and the level of potassium found in hydrocele, pleural and ascitic fluids may be explained in this way.

2. The potassium in the cerebrospinal fluid was always found to be lower than that in the serum, and consequently lower than that found in serum-ultrafiltrates or effusions. So far as potassium is concerned, therefore, the C.S.F. does not represent a serum-ultrafiltrate.

We take this opportunity of thanking all those who have from time to time allowed their cases to be investigated or who have helped with the collection of material.

One of us (E. W.) is indebted to the Medical Research Council for a full-time personal grant, and the other (R. A. McC.) for a part-time grant.

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CXLVIII. A STAINLESS STEEL HIGH-PRESSURE ULTRAFILTER.

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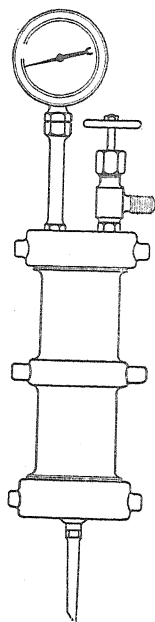
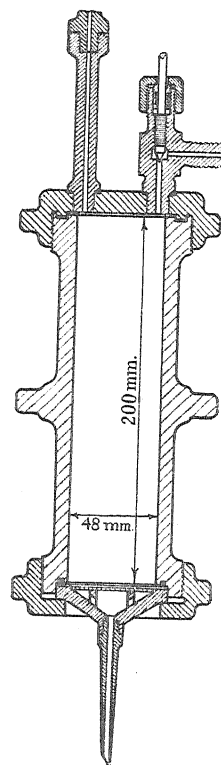
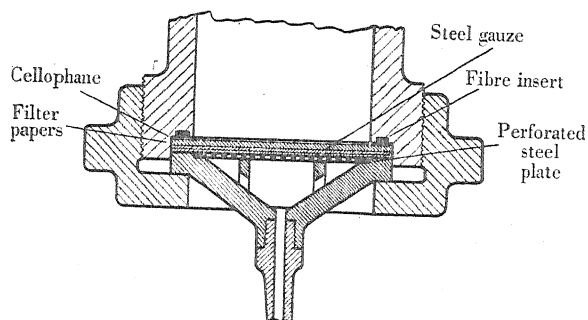
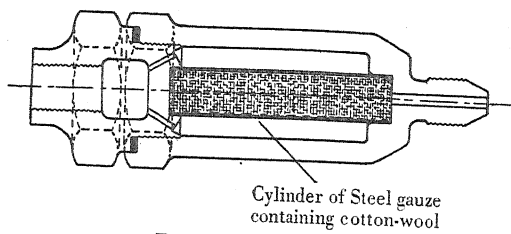
(Received July 1st, 1933.)

MANY biochemical processes involve tedious filtrations which are often rendered more difficult by the impossibility of removing very fine particles by means of filter-paper. Solutions containing impurities in fine suspension which are not removed on centrifuging at 4000 r.p.m. can usually be clarified by passage through suitable filters. Application of reduced pressure is, however, contraindicated in the case of solutions of capillary active substances such as proteins, on account of frothing which inevitably results in contamination of the filtrate by coagulated protein (Ramsden phenomenon) and also because of the excessive time required for filtration of considerable volumes of solution.

In order to facilitate the preparation of certain proteins which is being undertaken at this Institute, an apparatus has been designed by means of which positive pressure filtration of liquids can be effected at pressures up to 120 atmospheres, the maximum pressure obtainable from commercial nitrogen cylinders. For clarifying liquids and for bacteriological purposes, Seitz "Entkeimungsschichten," size 6, or suitable large-pored collodion membranes may be used with this apparatus, while for high-pressure ultrafiltration circles of cellophane Nos. 300 or 600 supported by 6 cm. circles of No. 42 Whatman filter-paper are suitable.

The apparatus described differs from those of Bechhold [1907], Aitken and Kay [1927], McBain and Kistler [1931] and Nicholas [1932] in that it is made of "Staybrite Austenitic" steel throughout, possible electrolytic effects due to the use of more than one metal thus being eliminated. A surface of austenitic steel appears to be more inert to protein solutions than the surfaces of brass, nickel, bronze or silver used by the above authors.

Various aspects of the apparatus are clearly shown in Figs. 1-4. The cylinder, of 370 cc. capacity, is closed at the top by a cap provided with a pressure gauge and needle valve to which is attached a specially designed cotton-wool filter (Fig. 4) which prevents entry of particles from the feed pipe and gas cylinder. This cap when tightened against the fibre insert in the upper rim of the cylinder need not be removed, since the filter is charged with liquid through the tapped hole carrying the pressure gauge. The filtering medium, supported by a stainless steel gauze (100 mesh), in turn supported by a perforated steel plate, is pressed tightly against the fibre insert in the bottom of the cylinder when the annular screw cap is tightened against the shoulder of the funnel which collects the filtrate. The upper rim of the funnel is ground perfectly flat in order to make a gas-tight joint. A locating dowel welded into the upper rim of this funnel

Fig. 1. ($\frac{1}{6}$ actual size.)Fig. 2. ($\frac{1}{4}$ actual size.)Fig. 3. ($\frac{1}{2}$ actual size.)Fig. 4. ($\frac{1}{2}$ actual size.)

prevents twisting of the filtering medium during assembly. Lugs welded into the circumference of the filter, as shown in Fig. 1, enable the whole to be assembled by means of a key and the socket of cast iron illustrated in Fig. 5. This socket is fixed to a block of hard wood screwed to the floor. When using Seitz filters the apparatus may, if desired, be autoclaved after assembling.

A smaller filtering area than might have been obtained for the same capacity of filter was chosen so that standard 6 cm. Seitz pads could be used, and so that small volumes of liquid could be dealt with without undue loss.

The filter is connected through the needle valve and dust filter to a nitrogen cylinder by means of a flexible copper pipe tested to a pressure of 5000 lb./sq. inch. It is quite safe to apply full cylinder pressure to the filter as it also is tested to 5000 lb./sq. inch. Filtration at 110 atmospheres through Seitz pads is very rapid and even through cellophane No. 300 about 100 cc. per day of a 0.6 % solution of bovine serum-albumin may be ultrafiltered.

This apparatus has proved invaluable for numerous purposes such as clarification of biological solutions unaffected by centrifuging, sterilisation of bacteriological media without application of heat and fractionation of colloidal solutions by ultrafiltration.

SUMMARY.

A high-pressure ultrafiltration apparatus constructed solely of stainless steel is described. Various applications of the apparatus are indicated.

Our thanks are due to Mr A. E. Rowsell for his skilled technical advice in the design of this apparatus. It was skilfully made by the Pulsometer Engineering Co., Reading, and it is a pleasure to express our gratitude to Mr J. B. Clewes of that firm for the great care taken by him in supervising its construction.

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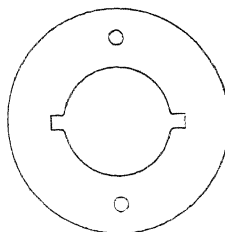


Fig. 5.

CXLIX. THE ACTION OF POLYHYDRIC PHENOLS ON UREASE; THE INFLUENCE OF THIOL COMPOUNDS.

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(Received July 1st, 1933.)

It has been reported by the writer [1932, 1], in a discussion on enzymes, that urease is poisoned by exceedingly minute quantities of polyhydric phenols and is completely protected from such poisoning by thiol compounds. This paper is concerned with a description of these phenomena.

Technique.

The experimental method adopted was that described in a previous communication [Quastel, 1932, 2] on the effects of dye-stuffs on urease. Briefly it consisted in exposing urease, prepared from soya bean or jack bean, to a dilute solution of a polyhydric phenol, at 45° and p_H 7.4, for 1 hour. Urea was then added to the mixture, and the ammonia formed at the termination of a subsequent hour's incubation at 45° was estimated by a suitable aeration method. The amount of enzyme usually taken was just sufficient to decompose 0.03 g. urea in 1 hour at 45° in the presence of phosphate buffer at an initial p_H of 7.4, i.e. the amount of $N/10$ ammonia liberated by the enzyme under these circumstances was 10 cc. Preliminary experiments were performed to determine the activity of any urease preparation, and the appropriate quantity was taken to fulfil the above conditions.

Urease from soya bean was prepared by shaking 1 part of soya bean meal with 9 parts of water for 30 minutes, centrifuging and diluting the centrifugate until the preparation had the desired enzymic activity. On certain occasions the enzyme was purified by passing CO_2 through the preparation so that proteins *etc.* were precipitated. Urease from jack bean was prepared in a similar manner, the amount of dilution of centrifugate being much greater (approximately 15-fold) to obtain a preparation of the same activity. A solution of crystalline urease was obtained from jack bean by Sumner's method. Solutions or suspensions of the enzyme were stored at 0° and frequently examined for change of activity. Fresh preparations were made as soon as any appreciable loss of activity occurred.

In a typical experiment 1 cc. of the urease preparation was added to 2 cc. $M/5$ phosphate buffer solution p_H 7.4. The phenol and any other substance¹ under investigation were added, and the volume was made up to 9 cc. with distilled water. The mixture was incubated at 45° for 1 hour. 1 cc. 3 % urea was then added and the incubation allowed to proceed at 45° for a further hour. At the

¹ All solutions of substances added to the enzyme were brought to p_H 7.4 at the commencement of the experiment.

end of this period the free ammonia was estimated on 1 cc. and compared with that obtained from urease treated under similar conditions but in the absence of the phenol (*i.e.* with a control experiment).

The toxic action of polyhydric phenols.

Representative results showing the effects of a number of polyhydric phenols, each at a final concentration of 1/10,000, on the activity of jack bean urease are recorded in Table I. The percentage inhibitions of the activity of the enzyme are given.

Table I. *Percentage inhibitions of activities of urease (jack bean) by polyhydric phenols 1/10,000.*

Catechol	98
Resorcinol	2
Quinol	99
Pyrogallol	96
Phloroglucinol	0
Gallic acid	96
Adrenaline	94
Protocatechuic acid	51
Protocatechuic aldehyde	15

The following facts are of note:

(1) Of the three dihydric phenols, resorcinol exhibits no inhibition of urease activity (within the experimental error). Catechol and quinol, on the other hand, are extremely toxic.

(2) Phloroglucinol is inactive, but pyrogallol is highly toxic.

(3) The inhibitory action of catechol is decreased by the introduction into the molecule of a —COOH group (protocatechuic acid) or a —CHO group (protocatechuic aldehyde).

Examination of Table II, in which the percentage inhibitions of the activity of jack bean urease by various concentrations of polyhydric phenols are noted, shows that catechol and quinol exert highly toxic actions at a concentration of 1 part in a million. Pyrogallol proves to be less toxic than catechol or quinol.

Table II. *Percentage inhibitions by various concentrations of polyhydric phenols (jack bean urease).*

	1/10 ⁴	1/10 ⁵	1/10 ⁶
Catechol	98	96	80
Quinol	99	95	80
Resorcinol	2	1	0
Gallic acid	96	57	10
Protocatechuic acid	51	11	0
Pyrogallol	96	91	31

The behaviour of adrenaline is of some interest. This substance is highly toxic to urease, but the toxicity varies greatly with the purity of the enzyme. For example, whereas adrenaline at a concentration of 1/10,000 effected 92 % inhibition of the activity of a jack bean preparation of urease, it only produced 40 % inhibition of a soya bean preparation of the same enzymic activity. The latter preparation contained a greater quantity of impurities in the form of proteins *etc.* than the jack bean preparation.

Similarly adrenaline at a concentration of 1/20,000 effected 96 % inhibition of the activity of a crystalline urease preparation and 36 % inhibition of activity of a jack bean preparation of the same enzymic strength.

This behaviour is very similar to that of many dyestuffs [Quastel, 1932, 2], whose toxicities towards urease are less in the presence of proteins than in their absence.

Catechol and quinol, however, even at a concentration of 1/100,000 are as toxic to soya bean as to jack bean preparations of the same enzymic content, which indicates the very powerful affinity of certain chemical groupings in urease for these dihydric phenols.

The influence of thiol compounds on the toxicity of polyhydric phenols.

If a thiol compound be added to a mixture of urease and a toxic phenol (at p_H 7.4), the inhibitory action of the latter is either diminished or eliminated. Such thiol compounds are cysteine, glutathione, thiolacetic acid and H_2S . Sodium hydrosulphite is also very effective. Illustrative results are shown in Table III.

Table III. *Percentage inhibitions of urease activity (jack bean) by catechol (1/10,000) in presence of various sulphur compounds (at p_H 7.4).*

Sulphur compound	Concentration %	Percentage inhibition
None	—	95
Cysteine	0.05	0
Glutathione	0.05	33
Thiolacetic acid	0.05	0
Dithiodiglycollic acid	0.05	93
Sodium hydrosulphite	0.02	18
H_2S water*	—	0

* Prepared by passing H_2S through distilled water for 5 minutes. 1 cc. of this was added to the mixture of enzyme and catechol, the final volume being 10 cc.

The percentage inhibition of urease activity effected by catechol or quinol decreases with increasing concentration of thiol compound present. Typical results are shown in Table IV.

Table IV. *Variation in percentage inhibitions of urease (jack bean) activity by catechol or quinol in the presence of varying concentrations of thiolacetic acid*.*

Concentration of catechol M	Concentration of thiolacetic acid M	% inhibition of activity
1.8×10^{-5}	—	84
"	1.1×10^{-5}	66
"	2.7×10^{-5}	45
"	5.4×10^{-5}	12
Concentration of quinol M		
1.8×10^{-5}	—	97
"	1.1×10^{-5}	76
"	2.7×10^{-5}	60
"	5.4×10^{-5}	22

* Present as the Na salt.

Similar results to those given in Tables III and IV may be obtained with soya bean urease and with a crystalline preparation of the enzyme. The toxicities of 1/10,000 concentrations of adrenaline, protocathechuic acid and gallic acid as well as those of catechol and quinol are entirely eliminated by 0.05 % thiolacetic acid. The oxidised form of this thiol compound—dithiodiglycollic acid—has no detoxicating action (Table III).

The action of potassium cyanide and of amino-acids.

It is well known that urease is poisoned by traces of metals, the toxicity being entirely removed by the presence of cyanide. Sumner has shown that the decrease in activity of crystalline urease in aqueous solutions is to be attributed to traces of metals (probably copper) in the water—the addition of a trace of cyanide to the water restores the full activity of the enzyme. Jacoby [1933] has recently made a study of the toxic actions of metals on urease and of the reactivating effects of cyanide and thiol compounds.

It seemed conceivable that the toxic effects of polyhydric phenols might be due to the presence of traces of metals. Recently the writer [1932, 2] has shown that hydroxylamine is highly toxic to urease but that the toxicity is entirely removed by the presence of 0.01 % potassium cyanide. Probably the hydroxylamine was contaminated with metals.

Investigations with catechol or quinol failed to show any reactivating action of potassium cyanide when this was added to a mixture of urease and the phenol. For example the activity of a crystalline urease preparation was destroyed to the extent of 97 % by the presence of 1/10,000 catechol. On addition of 0.01 % potassium cyanide the inhibition of activity became 96 %. When a much more dilute solution of catechol was used a very slight protective action of the cyanide was found, but with quinol as the toxic agent the writer has failed to find any protective action of cyanide (see Table V).

Table V. *Percentage inhibition of urease (jack bean) activity by catechol and quinol in presence of KCN and amino-acids.*

	Catechol 1/500,000	Quinol 1/100,000
Control	89	100
KCN (0.01 %)	78	100
Glycine (0.1 %)	61	100
Aspartic acid (1 %)	35	100

It appears very unlikely from these results that metallic impurities are responsible for the toxicity of catechol or quinol—a conclusion supported by the fact that purification of catechol by repeated sublimation failed to show an appreciable reduction in toxicity.

It has been shown by the writer [1932, 2] that amino-acids and amines afford protection to urease against the toxic effects of many dyestuffs. With very dilute solutions of catechol such protection can also be observed with glycine or aspartic acid, but the degree of protection is far smaller than in the case of the dyestuffs. Typical results are shown in Table V.

With a concentration of catechol and quinol of the order of 1/100,000 no protective action by amino-acids has been observed. Methylamine (*M*/30) which protects urease against the toxic activity of brilliant green (1/50,000) failed to show any protective action against 1/500,000 catechol.

These results show that the affinity of the toxic dihydric phenols for urease is far greater, under comparable conditions, than those of the most toxic dyestuffs yet investigated. This particularly high affinity is shown also in the fact that the presence of proteins such as those of egg-white or serum fail to protect urease from the toxic action of catechol (1/10,000).

It is, however, a fact of some interest that whereas egg-white does not protect urease (jack bean) against catechol, boiled egg-white is very effective.

E.g. % inhibition of urease by 1/500,000 catechol in presence of 1 cc. 10 % egg-white¹ is 89; % inhibition of urease by 1/500,000 catechol in presence of 1 cc. 10 % boiled egg-white is 7.

The most likely explanation of this phenomenon in view of the protective effects of thiol compounds already described is that the action of boiled egg-white is due to the liberated thiol groups.

Mode of action of catechol or quinol.

If the toxicities of catechol or quinol are due to their hydroxyl groups it is difficult to understand the mechanism of action of thiol compounds or of sodium hydrosulphite in producing a reactivation. Since the possibility of metallic compounds being responsible for the toxicity of the dihydric phenols is remote, it seems likely that the toxicity may be due to the presence of oxidation products of the phenols, these oxidation products being reduced by thiol compounds or sodium hydrosulphite to inert compounds. Two oxidation products suggest themselves: (a) hydrogen peroxide, (b) the quinone corresponding to the dihydric phenol.

(a) *Toxicity of hydrogen peroxide.* Hydrogen peroxide has a powerful inhibitory effect on the activity of urease, but a comparison of the toxicities of hydrogen peroxide and catechol at equivalent concentrations shows the latter to be the more toxic agent (see Table VI).

Table VI. *Percentage inhibition of urease (jack bean) activity by catechol, hydrogen peroxide and "hyperol".*

	Concentration M	% inhibition
Catechol	1.8×10^{-5}	85
Hydrogen peroxide	1.7×10^{-4}	28
"Hyperol" "	4.4×10^{-4}	52
	2.1×10^{-3}	39

"Hyperol" (an equimolecular compound of urea and hydrogen peroxide) is also less toxic than catechol. It follows that the toxicity of the latter cannot be due to the presence of traces of hydrogen peroxide in the aqueous solution of the dihydric phenol.

(b) *Toxicity of quinone.* On comparing the toxicities of quinone and quinol at equivalent concentrations it was found that the former had the greater inhibitory action (see Table VII).

Table VII. *Percentage inhibitions of urease activity by quinol and quinone.*

	Concentration	% inhibition
Quinol	1/2,500,000	64
Quinone	1/2,500,000	98
"	1/5,000,000	57

Quinone reduced the activity of urease (jack bean), under the experimental conditions given, over 50 % at a concentration of 1 part in 5 millions. Considering this very high activity of quinone it is not unreasonable to assume that the toxicity of aqueous quinol solutions may be due to the presence of traces of quinone. Quinol, even at very high dilutions, may well give rise to concentrations of quinone of the order of 1 part in 5 millions, since exposure of the solutions

¹ Prepared by adding 1 cc. fresh egg-white to 9 cc. water.

to atmospheric oxygen takes place freely under the experimental conditions employed. Experiments under anaerobic conditions have been carried out with a view to determining whether quinol was less toxic under these conditions, but the results were not satisfactory and mostly negative, owing, it is believed, to the difficulty of removing all traces of oxygen and of obtaining a specimen of quinol which could be assumed at the outset to be free from traces of quinone.

If the toxicity of quinol or catechol is due to the presence of the corresponding quinone in the aqueous solution and the protective action of thiol compounds is due to reduction of the quinone, it would follow:

(a) That the toxicity of a mixture of catechol and hydrogen peroxide should be greater than the sum of the individual toxicities owing to the production (by interaction of the peroxide with catechol) of a quinone more toxic than the hydrogen peroxide.

(b) That the amount of thiol compound necessary to protect urease from the toxic action of quinone should be greater than that necessary to protect urease from the toxic action of an equivalent concentration of quinol.

These predictions were verified by experiment. The following are typical results.

1. *Toxicity of a mixture of catechol and "hyperol" towards soya bean urease (purified by CO₂ precipitation).*

- | | |
|--|----|
| (a) Percentage inhibition of activity due to 1/5,000,000 catechol | 30 |
| (b) Percentage inhibition of activity due to 1/50,000 "hyperol" | 5 |
| (c) Percentage inhibition of activity due to a mixture of
1/5,000,000 catechol and 1/50,000 "hyperol" | 87 |

2. *Comparison of the protective actions of thiolacetic acid against the toxic effects of quinol and quinone (jack bean urease).*

- | | |
|---|-----|
| (a) Percentage inhibition of activity due to 1/10,000 quinol | 99 |
| (b) Percentage inhibition of activity due to a mixture of
1/10,000 quinol and 1/20,000 thiolacetic acid ¹ | 12 |
| (c) Percentage inhibition of activity due to 1/10,000 quinone | 100 |
| (d) Percentage inhibition of activity due to a mixture of 1/10,000
quinone and 1/20,000 thiolacetic acid | 100 |

On increasing the thiolacetic acid concentrations in (b) and (d) to 1/5000 the percentage inhibitions fell to 3 % in (b) and 14 % in (d).

DISCUSSION.

The evidence as a whole appears to be in favour of the view that the high toxicities of catechol and quinol towards urease are due to the presence of the corresponding quinones in the aqueous solutions of the dihydric phenols. The powerful protective effects of thiol compounds can then be explained as due to reduction of the quinones to the dihydric phenols². Presumably the toxicities of other polyhydric phenols are explicable on a similar basis.

The results are of interest in indicating the existence of substances, other than the metals, which are exceedingly toxic to urease and whose effects can be

¹ Present as Na salt.

² Baudisch and Dyer [1933] have recently shown that *o*-quinone is reduced by cysteine to catechol.

eliminated by the presence of thiol compounds. The fact that the dihydric phenols and their corresponding quinones are widespread in the biological kingdom lends biological significance to these results and points to the possibility that tissue extracts may well contain natural inhibitors to enzymes—quite apart from metals—whose effects are diminished or eliminated by sulphydryl compounds.

SUMMARY.

1. Of the three dihydric phenols, catechol and quinol are exceedingly toxic to urease but resorcinol is without action. Quinol at a concentration of 1 part in 2 millions will remove over 50 % of the activity of urease under the experimental conditions employed.

2. Adrenaline, protocatechuic acid, protocatechuic aldehyde, gallic acid and pyrogallol are toxic to urease but less so than catechol. Phloroglucinol is without action.

3. The toxicity of catechol or quinol at concentrations as low as 1/100,000 is not affected by the presence of potassium cyanide or amino-acids. This distinguishes the toxicity of catechol or quinol from that of metals or that of dyestuffs. The presence of protein (serum or egg-white) does not protect urease from catechol. Boiled egg-white has a protective action (due probably to liberated thiol groups).

4. Thiol compounds (cysteine, glutathione, thiolacetic acid, H_2S) and sodium hydrosulphite diminish or eliminate the toxicity of polyhydric phenols to urease.

5. Hydrogen peroxide and "hyperol" though very toxic to urease are less so than catechol.

6. Quinone is more toxic to urease than quinol.

7. Evidence is given to show that the toxic effects of catechol and quinol are probably due to the presence of the corresponding quinones in the aqueous solutions of these dihydric phenols, and that the protective action of thiol compounds is due to reduction of the quinone to the corresponding dihydric phenol.

The writer is indebted to the Medical Research Council for a grant towards the equipment of this laboratory.

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CL. METABOLISM OF NORMAL AND TUMOUR TISSUE.

IX. AMMONIA AND UREA FORMATION.

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(Received July 1st, 1933.)

As has been indicated in Part VIII of this series [Dickens and Greville, 1933, 1], the oxidation of protein by animal tissues may lead to different values of the respiratory quotient according to the nature of the nitrogenous end-products. The calculation of the theoretical values of R.Q. from protein oxidation is difficult, but arbitrary values showing the order of R.Q. to be expected may be derived using Loewy's [1911] figures for the analysis of meat protein and assuming the complete elimination of the protein-nitrogen as ammonia, urea or uric acid. When this is done the values obtained are approximately 0.94, 0.8 and 0.7 respectively. If, then, protein oxidation becomes an appreciable part of the total aerobic metabolism, the R.Q. may be greatly altered in a direction determined by the nature of the nitrogenous end-product, so that for the interpretation of the R.Q. it is important to establish the extent and nature of the protein oxidation and to assess its influence on the respiratory measurements.

Whilst numerous estimations of ammonia formation in isolated animal tissues have appeared, relatively few of them apply to the conditions of our experiments, and in still less has any correlation with the respiratory exchange been attempted. In the present paper we record estimations of NH_3 formation under the conditions previously employed for the classification of tissues according to their level of R.Q. in glucose-containing media [Dickens and Šimer, 1930, 2; 1931, 2]. The effect of sugar deprivation on the metabolism has already been described [Dickens and Greville, 1933, 1, 2] and we therefore include measurements of NH_3 formation in glucose-free media. As an example of the effect of other sugars we have extended the measurements to the behaviour of tissues in fructose. In all these cases simultaneous measurements of respiratory and glycolytic exchange have been carried out on the same portions of tissue as those used for the ammonia measurements. In addition, in a few selected cases the urea formation has been simultaneously estimated.

Methods.

The respiratory measurements were made by the methods of Dickens and Šimer in phosphate- [1930, 1] and in bicarbonate-Ringer solution [1931, 1]. Thin slices of tissue, prepared immediately after the death of the animal, were well rinsed by suspending in oxygenated sugar-free Ringer solution for 5-10 minutes. Equal weighed portions (usually 70-100 mg. wet weight) were then transferred to Ringer solution (1.5 to 3 cc.) contained in the manometric vessels, provided with side-bulbs containing a slight excess of 2N HCl. The initial p_{H}

of the Ringer solution was 7.4 and when glucose and fructose were added their concentration was 0.2 %. For each experiment two such vessels were used, placed side by side in the thermostat at 37°. After shaking for 10 minutes the acid was added from the bulb to one portion, which was removed from the bath after the necessary measurements of pressure change giving the CO₂ content, and the contents were used for determination of the preformed NH₃. The respiratory experiment was continued with the other portion of tissue, which was treated similarly at the end of the experiment. The difference of ammonia contents, corrected if necessary for any difference in the dry weights of the two portions of tissue, gave the ammonia formation during the experimental time of 2 to 5 hours. A similar procedure was used for the anaerobic experiments, which were made in bicarbonate-Ringer solution saturated with nitrogen containing 5 % CO₂.

Ammonia was estimated by the method of Stanford [1923] with the modifications recommended by Watchorn and Holmes [1927] and Bülow and Holmes [1932]. Deproteinisation, by addition of a slight further excess of *N* HCl (0.1 cc.) and 10 % sodium tungstate (0.5 cc.), was used in some experiments; and whilst this was found advisable with some tissues, particularly with spleen with which without deproteinisation foaming may occur during distillation, its use did not ever materially affect the ammonia values obtained. Ammonia-free water and tested reagents were used, and no ammonia was allowed in the laboratory. With these precautions the results of duplicate analyses agreed well, particularly when the ammonia-formation was pronounced. For very small amounts (under 4γ, say) the method is probably not sufficiently accurate; though provided the quantities of tissue are sufficient, as in our experiments, such experiments are of value in showing that the amount of ammonia formation is negligible in relation to the total metabolism.

Figures for "preformed" ammonia estimations are often lacking from publications on this subject. That they are frequently large, indicating a far higher ammonia content than that of the blood, for example, has been pointed out for brain-tissue by Schwarz and Dibold [1932]. Our experience confirms this; and in some of the other tissues quite high values were obtained (Table I). On the origin of this ammonia, on the extent to which it is traumatic, we express no opinion. Determinations of the ammonia contents of different slices of the same tissue, treated similarly as in our experiments, agree quite well; and nothing suggests that any uncertainty as to the "preformed" value is considerable enough to cause significant error in our measurements of the change in total ammonia.

Urea was estimated by the manometric urease method of Krebs and Henseleit [1932], which is particularly suitable for the detection of very small quantities of urea in these experiments, since its accuracy is unaffected by the presence of ammonia. Instead of soya bean we used powdered jack bean ("Arlco") with excellent results, as shown by analysis of standard urea solutions.

Results.

The data showing the extent of ammonia formation are collected in Table I, where the ammonia formation in bicarbonate- (B) or phosphate-Ringer (P) solution is given in γ NH₃ per mg. dry weight of tissue. The actual amounts of NH₃ measured were usually from 5 to 15 times as great, according to the weight of tissue used. The simultaneously determined values of respiration (Q_{O_2} , R.Q., aerobic and anaerobic glycolysis ($Q_M^{O_2}$ and $Q_M^{N_2}$) are given in Table II for the same pieces of tissue as those used for ammonia estimation. Table III shows

Table I. *Ammonia formation.*

Ammonia-nitrogen, 10 ⁻³ mg. per mg. dry tissue											
Exp. No.	Medium	Time (hours)	Glucose			Fructose			No addition		
			Pre-formed	Final	Diff.	Pre-formed	Final	Diff.	Pre-formed	Final	Diff.
AEROBIC EXPERIMENTS.											
Retina (rat):											
1	P	5	3.6	3.2	-0.4	—	—	—	2.3	3.2	0.9
2	B	5	0.34	0.84	0.50	0.17	0.21	0.04	—	—	—
3	P	5	—	—	—	1.1 1.5	3.0 3.0	1.9 1.5	4.7	6.4	1.7
Testis (rat):											
4	B	3	0.07	0.08	0.01	0.10	0.09	-0.01	0.32	0.72	0.40
5	B	3	0.28	0.23	-0.05	—	—	—	0.23	0.61	0.38
Kidney (rat):											
6	B	3	0.82	2.73	1.91	0.60	2.01	1.41	0.53	3.67	3.14
7	B	3	0.12	2.55	2.43	0.16	1.52	1.36	0.18	2.72	2.54
8	B	3	0.40	0.79	0.39	0.29	0.67	0.38	0.26	3.30	3.04
Spleen (rat):											
9	B	3	1.03	1.10	0.07	1.40	1.82	0.42	0.71	1.65	0.94
10	B	3	—	—	—	0.59	0.59	0.00	0.40	2.08	1.68
11	B	2.25	0.33 0.36	0.64 0.63	0.31 0.27	—	—	—	0.56	2.38	1.82
Liver (rat):											
12	B	4.08	—	—	—	0.18 0.13	0.20 0.22	0.02 0.09	0.23	0.14	-0.09
13	B	3	0.38	0.18	-0.20	0.42	0.27	-0.15	0.12	0.22	0.10
Embryo (chick)*:											
a 14	B	4	0.64	0.75	0.11	0.51	0.79	0.28	0.54	1.65	1.11
b 15	P	4.17	—	—	—	0.72	0.70	0.0	—	—	—
c 16	B	3.5	0.30	0.34	0.04	0.16	0.66	0.50	0.48	1.00	0.52
d 17	P	3.5	0.50	0.74	0.24	0.52	0.78	0.26	0.51	1.21	0.70
e 18	P	3	—	—	—	0.89	0.99	0.10	0.59	1.65	1.06
f 19	P	4.25	—	—	—	0.34	0.82	0.48	0.25	2.14	1.89
Embryo (rat)*:											
g 20	B	3	0.30	0.30	0.00	0.25	1.10	0.85	0.0	1.70	1.70
Yolk-sac (rat):											
21	B	3.87	0.29	0.32	0.03	0.21	0.30	0.09	—	—	—
22	B	3	—	—	—	—	—	—	0.74	0.87	0.13
23	B	3.17	0.32	0.78	0.46	—	—	—	0.47	0.71	0.24
Jensen sarcoma (rat):											
24	B	3	0.47	0.53	0.06	—	—	—	0.16	1.74	1.58
25	B	3	0.15	0.14	0.01	0.07	0.28	0.21	0.23	0.23	0.00
26	P	2.5	—	—	—	0.22	0.58	0.36	0.29	2.52	2.23
27	B	2.5	0.28	0.35	0.07	—	—	—	0.35	1.91	1.56
28	B	3	—	—	—	0.51	1.06	0.55	0.60	2.78	2.18
ANAEROBIC EXPERIMENTS.											
Kidney (rat):											
6	B	3	0.96	1.68	0.72	0.47	1.30	0.83	0.56	0.81	0.25
7	B	3	—	—	—	—	—	—	0.23	0.23	0.00
8	B	3	0.42	1.97	1.55	0.55	1.60	1.05	0.21	0.16	-0.05
30	B	2	0.36	0.26	-0.10	—	—	—	0.21	0.75	0.54
Testis (rat):											
5	B	0	0.71	—	—	—	—	—	0.49	—	—
		1	—	—	—	—	—	—	—	0.82	0.33
		3	—	0.86	0.15	—	—	—	—	1.42	0.93
Liver (rat):											
13	B	3	0.34	—	—	0.36	0.50	0.14	0.37	0.21	-0.16
12	B	4	—	—	—	—	—	—	1.0	1.10	0.10
29	B	3.33	0.51 0.30	0.72 0.43	0.21 0.13	—	—	—	—	—	—
Spleen (rat):											
9	B	3	—	—	—	—	—	—	0.65	1.40	0.75
10	B	3	—	—	—	0.54	1.75	1.21	2.30	3.50	1.20
Jensen sarcoma (rat):											
24	B	3	—	—	—	—	—	—	0.35	0.87	0.52
25	B	3	1.01	0.71	-0.30	0.40	0.89	0.49	0.57	0.41	-0.16
26	B	2.5	—	—	—	—	—	—	0.28	0.86	0.58

* Embryo dry weights: a, 6.0; b, 5.1; c, 7.6; d, 7.6; e, 5.8; f, 8.3; g, 6.8 mg.

Table II. *Respiration, respiratory quotient and glycolysis.*

Exp. No.	Medium	Time (hours)	Glucose				Fructose				No addition					
			R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$		
Retina:																
1	P	5	—	24.5	—	—	—	—	—	—	—	—	—	—	—	—
2	B	5	0.94	20.3	22.5	—	0.94	17.2	0.3	—	—	—	3.0	—	—	—
3	P	5	—	—	—	—	1.02 0.99	14.6 16.2	—	—	—	—	4.5	—	—	—
Testis:																
4	B	3	0.88	10.2	6.8	—	0.82	8.5	2.1	—	0.82	4.8	1.8	—	—	—
5	B	3	0.93	6.9	4.2	—	—	—	—	—	0.76	3.1	0.9	—	—	—
Kidney:																
6	B	3	0.87	24.0	-0.9	4.8	0.92	24.6	0.0	1.3	0.84	17.3	-1.7	1.1	—	—
7	B	3	0.84	19.3	0.0	1.8	0.91	27.6	0.1	—	0.80	19.0	-1.1	2.8	—	—
8	B	3	0.87	22.2	2.0	7.6	0.89	24.9	0.2	2.8	0.79	18.9	-0.6	1.3	—	—
30	B	2	—	—	—	3.7	—	—	—	—	—	—	—	—	—	1.3
Spleen:																
9	B	3	0.92	10.8	4.8	—	0.81	10.5	3.6	—	0.83	8.9	0.3	6.6	—	—
10	B	3	0.89	11.0	5.3	—	0.85	12.7	4.0	5.6	0.83	9.0	0.6	4.4	—	—
11	B	2.25	0.85 0.86	17.0 14.6	6.7 6.5	—	—	—	—	—	—	—	—	—	—	—
Liver:																
12	B	4.08	—	—	—	—	0.79 0.77 0.78	8.5 7.4 7.0	2.9 3.5 3.0	4.0 3.6	0.70	7.8	2.0	1.4	—	—
13	B	3	0.67	7.3	2.8	1.7	—	—	—	—	0.58	8.6	2.1	1.5	—	—
Chick embryo:																
14	B	4	0.95	10.9	4.5	—	0.94	8.1	-0.6	—	0.91	8.0	-0.7	—	—	—
15	P	4.17	—	13.3	—	—	—	12.1	—	—	—	11.1	—	—	—	—
16	B	3.5	1.02	9.1	2.4	—	0.89	8.5	0.0	—	0.87	7.5	0.0	—	—	—
17	P	3.5	1.02	10.3	—	—	0.95	9.1	—	—	0.95	9.0	—	—	—	—
18	P	3	—	—	—	—	0.96	9.3	—	—	0.87	9.2	—	—	—	—
19	P	4.25	—	—	—	—	0.87	9.6	—	—	0.89	8.3	—	—	—	—
Rat embryo:																
20	B	3	0.97	11.1	5.2	—	0.95	11.4	0.0	—	0.78	9.2	0.7	—	—	—
Rat yolk-sac:																
21	B	3.9	—	(11.0)	—	—	0.98	11.0	1.1	—	0.91	11.4	0.3	—	—	—
22	B	3	—	—	—	—	—	—	—	—	0.80	11.0	1.1	—	—	—
23	B	3.17	1.05	10.3	2.4	—	—	—	—	—	0.92	8.9	1.1	—	—	—
Jensen rat sarcoma:																
24	B	3	0.81	13.6	11.1	—	—	—	—	—	0.67	10.4	0.4	1.2	—	—
25	B	3	—	—	—	21.2	0.76	11.3	6.6	10.7	0.73	12.4	0.4	1.8	—	—
26	P	2.5	0.85	10.6	—	—	0.87	13.1	—	—	0.81	12.8	—	1.6	—	—
27	B	2.5	0.83	9.3	9.3	—	—	—	—	—	0.73	9.1	0.7	1.4	—	—
28	B	3	—	—	—	—	0.81	14.1	5.6	—	0.78	12.6	0.1	—	—	—

Table III. *Urea formation*.*

Exp. No.	Time (hours)	Glucose				Fructose				No addition					
		R.Q.	Q_{O_2}	$Q_M^{O_2}$	Q_{urea}	R.Q.	Q_{O_2}	$Q_M^{O_2}$	Q_{urea}	R.Q.	Q_{O_2}	$Q_M^{O_2}$	Q_{urea}		
Chick embryo (5th day):															
31	B	4	—	9.8	—	+0.1	0.85	10.4	0.4	0.0	0.84	8.7	-0.3	0.0	
32	P	4	—	—	—	—	0.96	9.7	—	0.0	0.94	8.2	—	+0.1	
Rat yolk-sac:															
33	B	4	1.04	12.0	2.3	+0.1	—	—	—	—	0.94	11.8	-0.4	0.0	
Jensen rat sarcoma:															
27	B	2.5	0.83	9.3	9.3	+0.2	—	—	—	—	0.94	11.9	0.0	—	
Rat liver:															
34	B	3	0.60	7.8	3.5	+0.2	0.70	9.0	3.4	+0.1	0.66	8.3	2.4	+0.3	

* $Q_{urea} = \frac{\text{mm.}^3 \text{ urea-CO}_2}{\text{mg. (dry) tissue} \times \text{hrs.}}$. (To express as mg. urea per mg. per hour, multiply by 0.269×10^{-3} .)

the results of simultaneously made estimations of urea. In this case the values of "preformed" urea are omitted as they were in all cases negligible [cf. Krebs and Henseleit, 1932].

From the figures given in Table III it is evident that in none of the cases examined (rat liver, embryo, yolk-sac, Jensen rat sarcoma) was the urea formation appreciable when compared with the total metabolism, under the conditions of our experiments. The effect on the R.Q. of retention of carbon as urea to the extent indicated in Table III would be hardly detectable. Naturally, these observations apply only to washed tissues in ordinary Ringer solutions. Under special conditions, as in the presence of fairly high concentrations of ammonia, the urea formation by isolated liver tissue may be very large, as Krebs and Henseleit have shown. It should be noted that these authors found that rat brain, retina, kidney, spleen, testis and yolk-sac do not produce measurable amounts of urea, even under conditions which are optimum for urea formation by liver. Taking our results in conjunction with these, we may probably neglect the possibility of urea formation by the tissues discussed in this paper.

Relationship of NH_3 formation and respiration. In order to correlate the observed values of respiration (Q_{O_2}) with the ammonia formation it is convenient to calculate the latter in similar units to the Warburg units for respiration: Q_{NH_3} is then expressed in $\text{mm}^3 \text{NH}_3$ (at 0° and 760 mm.) formed per mg. dry weight of tissue per hour (10^{-3} mg. $\text{NH}_3 = 1.32 \text{ mm}^3$). These values, and their means, have been collected in Table IV.

These figures, taken in conjunction with the respiratory measurements already given, give an idea of the extent to which protein oxidation leading to ammonia may contribute to the total metabolism. Taking the figures used by Loewy [1911] for the analysis of a typical protein, and assuming complete elimination of all N as ammonia with complete oxidation of the remainder of the molecule, the calculation of R.Q. for pure protein oxidation is as follows:

100 g. protein	C 58.4	H 7.3	O 22.7	N 16.65	S 1.02
Deduct for NH_3		3.6		16.65	
" S-oxidation			1.5		1.02
" intramolecular H_2O		2.65	21.2		
Residue for oxidation	C 58.4	H 1.05			
Requires: O_2	$155.8 + 8.4 + 1.5 = 165.7 \text{ g.} = 116 \text{ l.}$				
Produces: CO_2	$214.2 \text{ g.} = 109 \text{ l.}$				
	$\text{R.Q.} = \frac{109}{116} = 0.94.$				

Oxidation of 1 mg. protein thus requires 1160 mm^3 oxygen and produces 1090 mm^3 carbon dioxide together with 0.202 mg. NH_3 ($= 266 \text{ mm}^3$). Hence the ratio

$$\frac{Q_{\text{O}_2}}{Q_{\text{NH}_3}} = \frac{\text{mols. oxygen consumed}}{\text{mols. ammonia produced}} = \frac{1160}{266} = 4.4 \text{ approx.}$$

for the complete oxidation of this protein to NH_3 , CO_2 , H_2O and SO_3 . Since the tissue proteins vary somewhat in composition and it is uncertain how far the oxidation follows the above course, it is clear that this value may only be used as an approximation, but as such it is a useful guide to the probable amount of ammonia-producing protein oxidation. In the right-hand columns of Table IV are given the individual and mean values of the ratio $Q_{\text{O}_2}/Q_{\text{NH}_3}$: in the calculation of the latter the mean Q_{O_2} of all experiments with the given tissue (Table II) has been divided by the mean Q_{NH_3} .

Inspection of these figures shows that the ammonia formation is almost always greatest when no sugar is supplied to the tissue. In sugar-free media

the amount of protein oxidation, if this be indicated by the Q_{O_2}/Q_{NH_3} ratio, may rise to about one-half of the total respiration (retina, kidney, spleen, Jensen rat sarcoma). In the ammonia-producing tissues, with the exception of kidney,

Table IV. *Relation of ammonia formation to respiration.*

Tissue	Exp. No.	Q_{NH_3}			$\frac{\text{mols. } O_2}{\text{mols. } NH_3} (=Q_{O_2}/Q_{NH_3})$		
		Glucose	Fructose	No addition	Glucose	Fructose	No addition
Retina	1	-0.13	—	0.29	(-190)	—	10.3
	2	0.16	0.01	—	127	1700	—
	3	—	{ 0.60 0.48	0.55	—	{ 24 34	8.2
	Mean*	0.02	0.28	0.42	Large	58	9
Testis	4	0.005	-0.005	0.21	2000	(-1700)	23
	5	-0.02	—	0.18	(-350)	—	17
	Mean	(-0.01)	(-0.005)	0.20	Large	Large	20
Kidney	6	1.02	0.75	1.67	23.5	32.8	10.3
	7	1.29	0.73	1.35	15.0	37.8	14.0
	8	0.21	0.20	1.62	106	124	11.7
	Mean	0.84	0.56	1.55	26	46	12
Spleen	9	0.04	0.22	0.50	270	48	17.8
	10	—	0.00	0.90	—	Large	10.0
	11	{ 0.22 0.19	—	1.29	{ 77 77	—	7.0
	Mean	0.12	0.11	0.90	104	105	10
Liver	12	—	{ 0.008 0.035	-0.035	—	{ 106 212	(-220)
	13	-0.11	-0.08	0.053	(-66)	(-87)	162
	Mean	-0.11	-0.01	0.01	(-ve)	(-ve)	(Large)
Chick embryo	14	0.044	0.11	0.44	250	74	18
	15	—	0.00	—	—	Large	—
	16	0.018	0.23	0.24	510	37	31
	17	0.11	0.12	0.32	94	76	28
	18	—	0.05	0.57	—	184	16
	19	—	0.18	0.71	—	53	11.7
	Mean	0.06	0.12	0.46	190	79	20
Rat embryo	20	0.00	0.45	0.91	Large	25.4	10.1
Rat yolk-sac	21	0.012	0.04	—	920	270	—
	22	—	—	0.07	—	—	160
	23	0.23	—	0.12	45	—	75
	Mean	0.12	0.04	0.10	89	270	104
Jensen rat sarcoma	24	0.032	—	0.84	425	—	12.4
	25	0.005	0.11	0.00	2400	103	(Large)
	26	—	0.23	1.42	—	57	9.0
	27	0.04	—	1.00	230	—	9.1
	28	—	0.29	1.16	—	49	10.9
	Mean	0.026	0.21	0.88	430	61	13

* The mean values of $\frac{\text{mols. } O_2}{\text{mols. } NH_3}$ given are the mean values of Q_{O_2} divided by the mean values of Q_{NH_3} .

the "protein-sparing" action of added sugar is apparent; for in the presence of glucose the amount of oxidation of nitrogenous bodies leading to ammonia formation is certainly small. If reckoned as protein oxidation it usually amounts to less than 5 % of the total oxygen uptake. "Protein-sparing" is also seen to

occur in the presence of fructose; and our experiments provide an interesting demonstration that the phenomenon of "protein-sparing" by carbohydrates applies to a wide variety of tissues and persists with isolated tissues *in vitro*.

The extent to which ammonia formation occurs is so different with different tissues that each must be considered separately. Under our conditions washed liver tissue produces barely appreciable quantities of ammonia, and a slight ammonia consumption sometimes occurs. It is highly probable that the latter is due to urea formation, which, as Krebs and Henseleit [1932] showed, proceeds more readily from ammonia in slices of surviving liver tissue than in any other tissue examined by them. The experiment (No. 34) in Table III gives an idea of the magnitude of urea formation in washed liver. It is roughly of the same order as the ammonia figures for the same tissue, that is to say too small for accurate measurement and negligible in its contribution to the total metabolism. Hence the low R.Q. found with washed liver slices is not due to urea formation, and, unless uric acid or some unconsidered nitrogen compound be formed, the liver does not derive its energy from oxidation of its own protein. Since isolated liver tissue does not apparently oxidise glucose from the medium to any great extent [Dickens and Greville, 1933, 1], it seems probable that liver uses a mixed oxidation of its own fat and carbohydrate (glycogen) reserves. The value of the R.Q. is in accordance with this view [Dickens and Šimer, 1930, 2; 1931, 2]. In the presence of fructose liver may show a large glycolysis, and the R.Q. is considerably raised. This shows that the power to oxidise carbohydrate is definitely present in liver tissue [Dickens and Greville, 1933, 1], although carbohydrate oxidation has never been observed to cover the whole respiration. Possibly by the use of strong fructose solutions the percentage carbohydrate oxidation could be further increased: this is being investigated.

Unlike liver, kidney shows a strong ammonia formation in all experiments in sugar-free media, only partially removed by addition of glucose or fructose. This is of particular interest since the kidney is considered by many workers to be the seat of nearly all the ammonia formation in the body [see Krebs, 1932]. Warburg, Posener and Negelein [1924], however, on the basis of the temperature coefficient, considered that ammonia elimination by isolated rat's kidney was not a chemical reaction but a mechanical washing-out of preformed ammonia from the tissue. They further stated that sugar did not affect the ammonia production. The evidence, however, was not convincing and was greatly weakened by the demonstration of Patey and Holmes [1929] and of Holmes and Patey [1930] that the ammonia production was much less under anaerobic conditions. These authors, however, using the differential Barcroft apparatus and chopped kidney were unable to show that deamination of added amino-acid (glycine) was accompanied by a comparable extra oxygen uptake; but Krebs [1932] has since shown that with tissue slices such an increase regularly occurs and has demonstrated beyond doubt an oxido-deaminase in kidney and its extracts. Holmes and Patey, however, were able to show that, contrary to Warburg, Posener and Negelein's statement, the aerobic ammonia production of kidney from its own nitrogen compounds was checked by the addition of glucose, whilst the anaerobic conversion was unaffected by sugar. The ammonia formation from added amino-acids was more sensitive to cyanide than that occurring without substrate, but like the anaerobic breakdown was unaffected by glucose. They concluded that two aerobic and one anaerobic mechanisms were at work, all resulting in ammonia production, and suggested that at least part of the anaerobic formation was from deamination of adenylic acid [*cf.* Embden and Schumacher, 1929].

Our observations on kidney slices support the results of Holmes and Patey, inasmuch as the ammonia formation from the tissue's own N compounds is diminished somewhat by addition of glucose, and more markedly by addition of fructose, which is more readily oxidised than glucose by the kidney; and also in that the anaerobic formation of ammonia (Table I) without added sugar is far less than the aerobic formation. With sugar, however, the difference is much less marked, though the anaerobic results are here so variable that our few measurements can lead to no definite opinion. From the comparison of the aerobic and anaerobic ammonia formation without added sugar, from the effect of sugar, particularly fructose, in increasing the R.Q. and the total respiration and at the same time lowering the ammonia formation, we see no reason to doubt that the ammonia production thus checked is due to oxidative deamination, presumably of protein, though possibly in part of amino-acids or of other protein breakdown products existing preformed in the tissue. The Q_{O_2}/Q_{NH_3} ratio for kidney (Table IV) suggests that some 30 % of the oxygen uptake of kidney tissue without sugar may be due to protein oxidation leading to ammonia. Fructose depresses this part of the oxidation to less than one-tenth of the total. Glucose has less effect, and kidney is the one tissue yet studied in which a fairly large part (perhaps up to a quarter) of the total respiration in glucose may be due to oxidation of protein or protein derivatives. Since Holmes and Patey found that the ammonia formation by kidney from added amino-acids is not affected by glucose addition, and since kidney is the only tissue which readily deaminates added amino-acids under aerobic conditions [Krebs, 1932] and is the only tissue which produces large quantities of ammonia during its respiration in glucose, it seems possible that it is the oxidative deamination of preformed amino-acids in the tissue which may be, at any rate in part, responsible for the persistence of aerobic ammonia production in the presence of glucose. Finally, this oxidation of N compounds leading to ammonia gives a possible explanation of the anomalously high R.Q. of kidney in glucose in comparison with its ability to convert glucose into lactic acid [Dickens and Šimer, 1930, 2].

The nervous tissues, brain and retina, having normally an almost pure carbohydrate oxidation, share the property of being dependent on an external supply of sugar for the continuance of their respiration [Loebel, 1925; Dickens and Greville, 1933, 1]. The effect of glucose and lactic acid on the ammonia formation of brain has been fully investigated by numerous authors [Warburg, Posener and Negelein, 1924; Loebel, 1925; Watchorn and Holmes, 1927; Bülow and Holmes, 1932], and we have not therefore included any of our observations in this paper. The ammonia formation of brain may be lowered somewhat in the presence of oxidisable metabolites (sugar, lactic acid), but frequently the absolute amount of ammonia produced is not markedly affected, as is seen from the data of Loebel [1925]. It is mainly the ratio of oxygen respired to ammonia produced that is affected in these tissues, this being lowered in absence of sugar to a value indicating that a considerable part of the residual oxygen uptake may be due to protein oxidation. This, therefore, is a somewhat special case. The total respiration is so dependent on the presence of sugar that it falls to one-third or less in its absence. At the same time the actual ammonia formation is hardly affected. An illusion of "protein-sparing" is thus produced, in that the protein fraction of the respiration is lessened by the presence of sugar. This effect is much less in evidence in most of the other, non-nervous, tissues examined, since their respiration is less affected by the presence or absence of sugar.

The behaviour of retina closely resembles that of brain. Both tissues have a fairly large preformed ammonia content. Our experiments with retina suggest

some decrease of ammonia production in presence of sugar, but here also an illusion of great protein-sparing is produced. The value of the ratio Q_{O_2}/Q_{NH_3} suggests high protein oxidation in absence of substrate, the protein oxidation becoming relatively much less in presence of either glucose or fructose, both of which support the respiration of retina [Dickens and Greville, 1933, 1]. It is hardly practicable to measure the R.Q. of retina without added substrate; but that of brain (rat, rabbit) in our experience remains near unity under these conditions in conformity with the view that protein is being oxidised to give ammonia. As a result of one experiment Loebel [1925] shows a fall of R.Q. to 0.86, but we find that it is unusual to encounter such a marked decrease.

Among tissues with less marked glycolytic and sugar-oxidising ability, testis shows a point of resemblance to brain in that its respiration falls considerably in absence of sugar. Some indication of a protein-sparing action of the sugars is given by the fall in ammonia excretion when the tissue is supplied with either glucose or fructose (Table IV). The anaerobic ammonia formation may, however, be also surprisingly large even in the presence of sugars, making interpretation of the results difficult. It is clear from the ammonia figures, however, that the metabolism in glucose or fructose does not consist to any appreciable extent of oxidation of protein to ammonia; and support is given to the view that fructose is oxidised by this tissue [Dickens and Greville, 1933, 1].

From Table IV it is seen that spleen has a high ability to form ammonia from its own N compounds, or possibly to some extent from those of the blood which invariably accompanies this tissue into the manometric vessels, even after careful rinsing. Here both glucose and fructose reduce to a low level the high aerobic ammonia elimination found in the absence of these sugars. This supports the R.Q. measurements in indicating an oxidation of glucose by this tissue and renders it probable that fructose is also oxidised to some extent even though the R.Q. is apparently not increased by its presence.

The embryonic tissues studied were the rat yolk-sac and the embryos of chick and rabbit. In the first-named tissue the ammonia formation was low in all cases, both in presence and absence of added glucose or fructose. There is therefore little oxidation of protein resulting in ammonia formation by rat yolk-sac under any of these conditions. As Table III shows, the urea formation is also negligible. Since in the absence of added substrate the respiration does not fall significantly, and the R.Q. remains at a relatively high level, it seems probable that the metabolism of the yolk-sac may be adequately supported by its own reserves of foodstuff, whose nature is not known [Dickens and Greville, 1933, 1].

The chick embryo resembles the rat yolk-sac in the persistence of the respiration at a nearly unchanged level in the absence of glucose or fructose. On the other hand, the ammonia estimations (Table IV) show that in this tissue in absence of sugar the ammonia formation is well-marked and may indicate a fairly extensive change of metabolism by the embryo to a protein oxidation, which is checked by addition of either glucose or fructose, both in phosphate and bicarbonate media. It thus appears that the embryo has at this age a considerable ability to turn to protein as a foodstuff, but only in the absence of oxidisable carbohydrates. It should be mentioned, however, that Needham [1932] considers that about 20 % of the respiration of the chick embryo in glucose is due to protein oxidation. The decreased ammonia production in the presence of fructose indicates that this sugar can diffuse into the embryo, and that its failure to cause lactic acid production in the embryo [Dickens and Greville, 1932] is not due to impermeability. Urea formation by the chick

embryo of 4-5 days' incubation is negligible under our conditions (Table III). The rat embryo of 13-14 days' gestation shows, like the chick embryo, a large ammonia production in absence of sugar, reduced by the addition of glucose or fructose.

The tumour tissue examined—Jensen rat sarcoma—presents several points of similarity to the embryonic tissues. Table IV shows that the ammonia formation in absence of substrate is large, both in phosphate and bicarbonate media. Since the aerobic ammonia production is much greater than the anaerobic, is practically abolished in glucose and is greatly reduced in fructose, we believe that the evidence is in favour of an oxidation of protein which may constitute up to 50 % of the total respiration in absence of sugar, whilst in the presence of sugar the protein oxidation falls to a low level. In all probability both glucose and fructose are oxidised by this tumour; but from the low value of the R.Q., and the fact that the R.Q. and the respiration are hardly affected by withdrawal of sugar, we infer that complete oxidation of carbohydrate accounts for only part of the respiration of the tumour cell. Under our experimental conditions, the urea formation by Jensen rat sarcoma was insignificant and quite unable to account for the characteristically low value of the R.Q.

Oxidative deamination of nitrogen compounds in the tissue can strictly be assumed only when the aerobic ammonia production far exceeds the anaerobic. This qualification has been kept in mind throughout, but it may be unduly strict. For the deamination observed by us with certain tissues, particularly spleen, under anaerobic conditions may possibly also be oxidative, at least in part, owing to the presence of hydrogen acceptors in the tissue; so that the existence of a large anaerobic deamination does not necessarily mean that the aerobic ammonia formation is not indicative of the oxidation of nitrogenous bodies.

Oxidative ammonia production may be due to the oxidation of nitrogenous bodies other than protein. Even if it is due to protein oxidation, this may not be complete. In either case, some of our conclusions are quantitatively altered. But the answer to the point which we set out to test is independent of these considerations: in a glucose medium the oxidation of nitrogenous bodies by all the tissues examined, except kidney, is so small as to have no material influence on the respiratory quotient. This is true in so far as can be judged from ammonia and urea formation. The possibility of protein oxidation under these conditions leading to the production of uric acid or other nitrogenous bodies has yet to be tested.

SUMMARY.

1. In the presence of glucose or fructose, and also in absence of added substrate, the ammonia formation by various washed animal tissues has been compared with the respiration and respiratory quotient, observations of all three quantities being simultaneously made on the same slice of tissue.
2. Little ammonia or urea was formed under our conditions by rat liver or yolk-sac either in presence or absence of sugar. Since the respiration and R.Q. of these tissues are not greatly altered by the presence of added sugar, it seems possible that their metabolism may be adequately supported by their foodstuff reserves, which in the yolk-sac are of unknown nature.
3. With kidney, spleen, Jensen rat sarcoma and embryo (chick, rat) the aerobic ammonia formation was large in absence of sugar, but was arrested by glucose, except with kidney, where it persisted at a reduced but still large value.
4. In all cases where glucose reduced the ammonia production, fructose had a similar effect. In kidney, fructose is more readily oxidised than glucose, and correspondingly it reduces the ammonia production to a greater extent.

5. In brain, retina and testis the amount of ammonia produced was small but in absence of glucose or fructose became large relative to the respiration, because of the large fall in the latter.

6. The "protein-sparing" action of glucose and fructose has thus been demonstrated for a variety of animal tissues, but with brain and retina it may be largely illusory.

7. Anaerobic ammonia elimination under the same conditions as in the aerobic experiments may be large, as in spleen and testis, but most often it is very much less than the aerobic production.

8. Urea formation by 5-day chick embryo and by Jensen rat sarcoma is so small that its effect on R.Q. measurements under our conditions can be neglected.

9. In general, in so far as can be judged from ammonia and urea formation, the assumption made in Part II of this series, that in a glucose medium the protein oxidation is so small as to have no material influence on the R.Q., has received experimental justification. It is probable that, of the tissues studied, only in kidney does the oxidation of nitrogenous bodies cover any large part of the total oxidations under the conditions of these experiments. The relatively high value of R.Q. shown by kidney may be due in part to the oxidation of protein by this tissue.

We express our thanks to Prof. E. C. Dodds for the interest he has taken in these experiments.

One of us (F. D.) is a member of the scientific staff of the Medical Research Council, the other (G. D. G.) is the holder of a Halley Stewart Trust Fund scholarship, and to both of these bodies we gratefully make our acknowledgments.

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CLI. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

X. THE EFFECTS OF LACTATE, PYRUVATE AND DEPRIVATION OF SUBSTRATE.

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(Received July 1st, 1933.)

THE anaerobic glycolysis of surviving tissue is not only decreased by general enzymic poisons (*e.g.* heavy metals, fluoride) and by more specific inhibitors (*e.g.* halogen-acetic acids, nitric oxide), but it is also affected by certain substances which are possible intermediates in carbohydrate metabolism. Thus glyceraldehyde inhibits the glucolysis of tumours [Mendel, 1929], pyruvic acid accelerates the glucolysis¹ of certain normal tissues [Mendel, Bauch and Strelitz, 1931], and lactate itself has been shown to reduce the anaerobic glycolysis of brain [Meyerhof and Lohmann, 1926, 1, 2]. The alteration of the rate of metabolism without the introduction of substances foreign to the animal body is an experimental method of considerable interest, and in the present paper we describe our experiences with its use.

Effect of lactate on the anaerobic glycolysis.

Meyerhof and Lohmann found that the anaerobic glucolysis of brain was reduced to about one-half by the addition of both *dl*- and *l*-lactates. As *l*-lactate had as great an effect as the natural *d*-lactate, these authors concluded that "bei dieser Beeinflussung der Glykolyse handelt es sich offenbar nicht um einen Prozess, der mit der physiologischen Rolle der Milchsäure zusammenhängt." It was felt that the matter merited further investigation, both because of the remarkable effect of the unnatural optical enantiomorph, and also because the suppression of the glycolytic process by lactate is of interest in the study of tissue respiration in lactate-containing media.

Experimental. The effect of added lactate on the anaerobic glucolysis of testis and of thin slices of rat brain and Jensen sarcoma has been measured (Table I). Zinc *d*- and *l*-lactates² were prepared by resolution through the zinc ammonium salts [Purdie and Walker, 1895], seeding with crystals of *d*- and *l*-salts prepared from meat extract and by resolution with morphine [Irvine, 1906] respectively. The purity of the preparations was checked by determinations of rotation and of water of crystallisation. The sodium salts were prepared by treatment of the zinc salts with sodium carbonate [see Meyerhof and Lohmann, 1926, 2]. The glucolysis was measured for periods up to 3 hours; in the first experiment the lactates were present from the start, but in the other experiments the

¹ For definition of terms glycolysis, glucolysis *etc.* see Dickens and Greville [1932, 1].

² In this paper the naturally occurring (sarcocactic) acid is referred to as *d*-lactic acid, and its salts as *d*-lactates.

Table I. *Effect of lactate on anaerobic glycolysis of rat tissues.*1. *Jensen sarcoma.*

Added lactate	0	0.019 M d	0.02 M dl
$Q_M^{N_2}$ { 1st hour	27.4	26.6	27.0
2nd hour	21.6	22.1	22.0
3rd hour	22.0	18.3	21.5

2. *Jensen sarcoma* (Fig. 1). Lactate added 20 mins. after first reading.

Added lactate	0	0.02 M d	0.02 M l
$Q_M^{N_2}$ { 1st period (20 mins.)	46.5	49.5	44.7
2nd period (40 mins.)	43.5	26.3	34.5
3rd period (40 mins.)	36.8	23.3	31.5
% decrease 2nd period	6.5	47	23
3rd period	26	53	29.5

3. *Testis.* Lactate added 20 mins. after first reading.

Added lactate	0	0	0.02 M d	0.02 M l	0.02 M dl
$Q_M^{N_2}$ { 1st period (20 mins.)	8.4	10.5	9.5	10.8	8.7
2nd period (40 mins.)	8.1	10.5	8.3	10.3	7.7
3rd period (60 mins.)	7.8	9.7	6.4	9.1	6.2
% decrease 2nd period	3.5	0	12.5	4.5	11.5
3rd period	7	7.5	33	16	29

4. *Brain cortex.* Lactate added 15 mins. after first reading.

Added lactate	0	0	0.02 M d	0.02 M l	0.02 M dl
$Q_M^{N_2}$ { 1st period (15 mins.)	21.2	24.4	20.0	25.2	15.6
2nd period (60 mins.)	14.9	19.2	13.3	19.3	11.3
3rd period (60 mins.)	13.1	17.2	10.7	17.4	9.2
% decrease 2nd period	30	21	33	23	28
3rd period	38	30	47	31	41

5. *Brain cortex.* dl-Lactate added 20 mins. after first reading.

Added lactate	0	0	0.02 M	0.02 M	0.05 M	0.10 M
$Q_M^{N_2}$ { 1st period (20 mins.)	12.2	12.9	10.8	14.4	12.3	9.0
2nd period (60 mins.)	6.2	7.3	5.2	6.4	4.3	2.8
3rd period (60 mins.)	4.7	5.2	3.2	4.4	2.0	0.8
% decrease 2nd period	49	43	52	56	65	69
3rd period	61	60	70	69	84	91

lactates were added at the stated time from a side-bulb. In order to eliminate the effect of glycolytic inequalities between different slices of the same tissue, we give for each vessel for various periods after the lactate addition the percentage decrease in glycolysis from the value in the period before the lactate addition. By comparison with the corresponding falling off in the control vessel to which no lactate was added the effect of the lactate may be assessed.

From the results in Table I we conclude that 0.02 M d-lactate may sometimes cause a decrease of about one-half in the glycolysis (Exp. 2) as in the experiments of Meyerhof and Lohmann; on the other hand, the effect of the added d-lactate may be small, as in Exps. 1 and 4. Inhibition occurs with all three tissues. Our experiments, in contrast to those of Meyerhof and Lohmann, do not show that l-lactate causes as great an inhibition as does d-lactate: in Exps. 2 and 3 the natural salt has about twice the effect of its optical enantiomorph. Exp. 5 shows how the inhibition of brain glycolysis increases with increasing concentration of added lactate. This experiment is

represented in Fig. 2, where corrected values of $Q_M^{N_2}$ are shown, equal for each vessel to the observed $Q_M^{N_2}$ divided by the ratio of the glycolysis in that vessel before lactate addition to the average glycolysis before lactate addition.

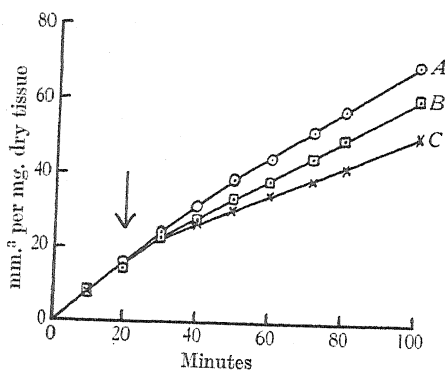


Fig. 1.

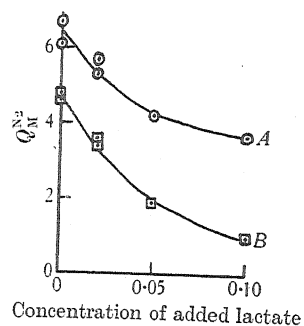


Fig. 2.

Fig. 1. Anaerobic glycolysis of Jensen rat sarcoma. A, control; B, 0.02 *M* sodium *l*-lactate added after 20 mins.; C, 0.02 *M* sodium *d*-lactate added after 20 mins.

Fig. 2. Rat brain cortex in glucose. A, second period; B, third period.

The inhibition of an enzyme reaction produced by addition of an end-product may be due to acceleration of the reverse reaction or to combination of the added substance with the enzyme system. The former may be excluded with glycolysis, for Lipmann [1927] found no anaerobic disappearance of lactic acid from minced muscle, and Burk [1932] has shown that when the free energy of neutralisation of lactic acid is negligible, the concentration of lactic acid would have to be 10^{20} *M* if an equilibrium with glycogen is to be attained. It thus seems probable that the lactate combines with the enzyme system.

Effect of substrate-deprivation.

Rosenthal [1930] showed that a spontaneous increase in the anaerobic fructolysis of Jensen rat sarcoma occurs about 40 minutes after the manometric vessels are put into the thermostat at 38° (Fig. 9). When glucose is substrate a spontaneous increase in $Q_M^{N_2}$ is not ordinarily observed, but we were able [Dickens and Greville, 1932, 2] to demonstrate its occurrence by introducing a preliminary period of anaerobiosis in absence of added glucose. Spontaneous anaerobic activation of glycolysis has so far been reported only in the Jensen sarcoma. In the experiments described in the present paper we applied to other tissues the technique of preliminary anaerobic substrate-deprivation which brought to light activation of glycolysis in the tumour tissue. Adding glucose to give a concentration of 0.2 %, we have tested normal tissues (rat brain, spleen, testis), another tumour tissue (Mill Hill fowl fibro-sarcoma), and an embryonic tissue (rat yolk-sac). With testis a slight spontaneous activation was detected. In the experiment represented in Fig. 6, $Q_M^{N_2}$ before glucose addition was 4.8: for 15 minutes after glucose addition it was 7.6, and then it rose abruptly to 9.8, which was maintained until the end of the experiment, 55 minutes later. No evidence of the occurrence of spontaneous activation in the other tissues was obtained (Figs. 4, 5, 7, 8). The spontaneous increase in fructolysis shown by the Jensen rat sarcoma occurs only within a certain range of fructose concentration

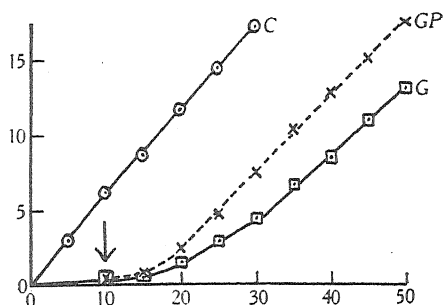


Fig. 3. Jensen rat sarcoma.

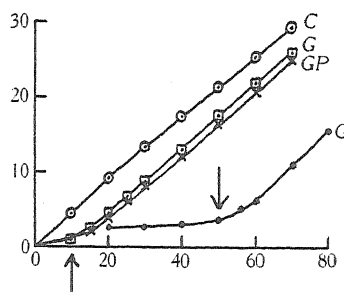


Fig. 4. Mill Hill fowl fibro-sarcoma.

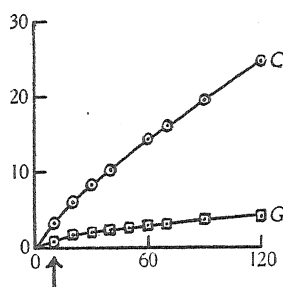


Fig. 5. Rat brain cortex.

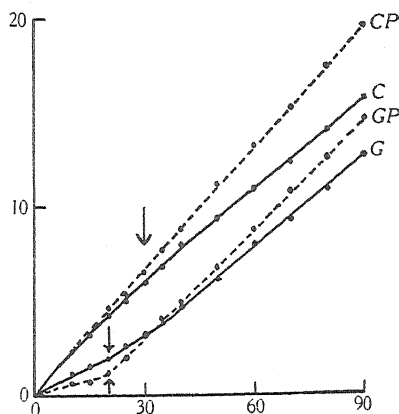


Fig. 6. Rat testis.

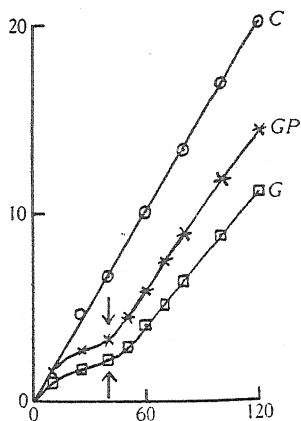


Fig. 7. Rat spleen.

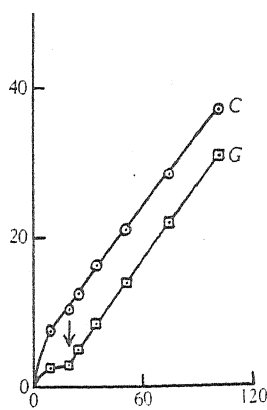


Fig. 8. Rat yolk-sac.

Figs. 3-8. Anaerobic glycolysis. Ordinates, $\text{mm}^3 \text{CO}_2$ per mg. dry tissue; Abscissae, time in minutes.

C = control (glucose present from beginning of experiment).

G = glucose added at time indicated by arrow.

GP = glucose + pyruvate added at time indicated by arrow.

CP = pyruvate added at time indicated by arrow to control (glucose present from beginning of experiment).

[Dickens and Greville, 1932, 3], and it was not observed at 28°, so that it is possible that the necessary conditions for the other tissues were not realised in these experiments. We therefore do not consider that spontaneous activation is a unique property of the Jensen sarcoma. The two processes which allow the observation of spontaneous activation, initial depletion of "activating" substance in the tissue and slow re-establishment of its optimum concentration, occur under the usual experimental conditions most readily with the Jensen sarcoma in fructose and to a lesser extent with the same tumour and with rat testis in glucose. That they occur in other tissues, that the glycolysis of these tissues also is regulated by an "activating" substance, is a possibility that still remains.

If for each tissue the glucolysis attained after a preliminary period of anaerobiosis in absence of added substrate is expressed as a percentage of the glucolysis of that tissue when glucose is present from the beginning of the experiment, the following values are obtained:

Tissue	Preliminary period in absence of substrate (mins.)	% recovery after glucose addition
Jensen rat sarcoma	20	92
Mill Hill fibro-sarcoma	20	106
Mill Hill fibro-sarcoma	60	112
Rat yolk-sac	30	110
Rat spleen	50	70
Rat testis	20	101
Rat brain cortex	20	12, 12, 11*

* Three different brains.

Very remarkable is the feeble recovery shown by brain cortex. We have indeed found that a very short anaerobic period in the absence of glucose will reduce the subsequent glucolysis of this tissue to one-third of the control value, as shown by the following experiment: brain slices were shaken in the bath for 25 minutes in glucose-free Ringer solution with oxygen and CO₂ in the gas space. Then nitrogen-CO₂ mixture was passed through the vessel for 3 minutes, after which glucose was immediately added from a side-bulb. $Q_M^{N_2}$ in the subsequent 30 minutes was 6.2. In a control experiment with glucose under anaerobic conditions from the start, $Q_M^{N_2}$ was 16.6. The 3-minute anaerobic substrate-deprivation thus caused a reduction of the subsequent glucolysis to 37 %. With the same brain a 25-minute preliminary period caused a reduction to 13 %. The preliminary aerobiosis involved in this experiment caused no reduction of the glucolysis; for when glucose was added immediately before nitrogen was passed, $Q_M^{N_2}$ was subsequently actually higher than in the control.

With brain, a preliminary period in absence of glucose has less effect on subsequent respiration in glucose than on subsequent anaerobic glucolysis. Thus a 40-minute preliminary aerobiosis followed by addition of glucose reduced the respiration in phosphate-Ringer solution to 50 % of the value in a control experiment in which glucose was present throughout. A 25-minute preliminary anaerobiosis in absence of sugar also reduced the respiration after glucose addition to about 50 % of the control value. It is remarkable that a preliminary anaerobic substrate-deprivation should affect subsequent respiration in glucose far less than subsequent anaerobic glucolysis.

The presence of 10^{-3} M sodium pyruvate (see below) does not cause brain glucolysis to recover any better from preliminary anaerobic substrate-deprivation.

Effect of pyruvate.

That sodium pyruvate in small concentrations causes an acceleration of the anaerobic glycolysis of brain, intestinal mucous membrane and liver was shown by Mendel, Bauch and Strelitz [1931]. Rosenthal [1932, 1] compared the activation of the anaerobic glycolysis of liver by pyruvate with the activation produced by preliminary aerobiosis, concluding that the two activation processes depend on the presence of the same factor in the liver, but that the "activator" produced by aerobiosis is not identical with pyruvic acid. Pyruvate also produces an activation with Jensen sarcoma.

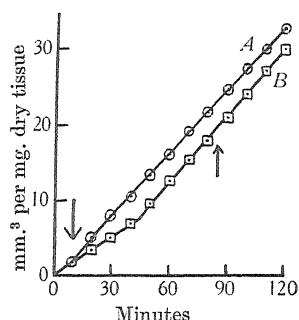


Fig. 9. Anaerobic fructolysis of Jensen rat sarcoma. A, pyruvate added at 10 mins. B, pyruvate added at 85 mins.

It was found [Dickens and Greville, 1932, 2] that its addition in 10^{-3} M concentration to this tissue before the spontaneous increase of anaerobic fructolysis had occurred caused an immediate increase in fructolysis, $Q_M^{N_2}$ thereafter remaining constant at about the value it would have reached by spontaneous activation, whilst addition of pyruvate after the spontaneous increase had no effect at all (Fig. 9). Also, after preliminary substrate-deprivation, an immediate rise of $Q_M^{N_2}$ to the final value occurs on adding glucose in the presence of pyruvate, the intermediate period being abolished (Fig. 3). It is seen from Fig. 6 that the same occurs with the spontaneous activation of testis glucolysis, the intermediate period being abolished in the presence of pyruvate. Thus under those conditions which allow the spontaneous activation to be observed, an identical activation can be caused prematurely on addition of pyruvate.

Rosenthal [1932, 2] also found an activation of tumour fructolysis by pyruvate and by certain substances which are reduced by tissues (methylene blue, Lauth's violet, Capri blue and potassium ferricyanide). Rosenthal's results differ from ours in that a greater percentage activation was caused by pyruvate than occurred spontaneously. We, on the other hand, invariably find that pyruvate raises $Q_M^{N_2}$ to practically the same value as is reached by the spontaneous increase, and that pyruvate addition after the occurrence of that increase has no effect. Further, pyruvate addition has no effect on the fructolysis or glucolysis of the Mill Hill tumour, which fails to show spontaneous activation. Nevertheless pyruvate definitely has an effect on other tissues in which spontaneous activation has not been observed. Thus with pyruvate present from the start Mendel, Bauch and Strelitz [1931] observed with brain increases of $Q_M^{N_2}$ up to three times the control values. We have, however, found that addition of 10^{-3} M pyruvate to brain after it has been producing lactic acid for some time in the thermostat may merely prevent the glucolysis from falling. Again, with testis (Fig. 6) pyruvate merely prevented $Q_M^{N_2}$ from falling from its initial value, and the addition of pyruvate simultaneously with the glucose after preliminary substrate-deprivation brought $Q_M^{N_2}$ to the same value. With spleen, addition of 10^{-3} M pyruvate did not increase the glucolysis (see also Fig. 7). Thus in our experience the effect of pyruvate addition is most often to keep the tissue at its maximum glycolysis. Liver must be regarded as an exception to this: in this tissue pyruvate, aldehyde or a suitable oxidising agent must be present in order that a glycolysis of any considerable magnitude may be attained [Rosenthal, 1932, 1]. One of the factors necessary for the attainment of the maximum glycolysis

seems to be the establishment of a suitable reducible system and possibly of a redox potential in the right range. Most tissues seem to be capable of creating these optimum conditions themselves, but with surviving liver some external agency is necessary.

SUMMARY.

1. The alteration of the glycolysis of tissues by methods not involving the addition of substances foreign to the animal body has been studied.

2. The inhibitory effect of lactates on the anaerobic glycolysis of brain, testis and tumour is very variable. *l*-Lactate has about half the effect of the natural *d*-lactate.

3. After 20 minutes' preliminary anaerobic substrate-deprivation the glycolysis of brain recovers only to about 12 % of the normal value, whilst with the other tissues tested (testis, spleen, rat yolk-sac, tumours) the recovery is almost always complete. With brain, preliminary anaerobic substrate-deprivation affects the glycolysis far more than the respiration.

4. The method of preliminary anaerobiosis in absence of added substrate has been applied to the detection of spontaneous activation of anaerobic glycolysis in various tissues. This has been observed only in the glucolysis and fructolysis of the Jensen rat sarcoma and in the glucolysis of rat testis. It is not supposed however that the potentiality for spontaneous activation exists only in these tissues.

5. The effect of small quantities of pyruvate on the glycolysis of tissues is discussed. In tissues and under conditions in which spontaneous activation is observed, an equal activation is achieved on addition of pyruvate. The view is expressed that the presence of pyruvate tends to keep the glycolysis of tissues at its maximum value.

It is a pleasure to acknowledge the interest taken by Prof. E. C. Dodds in these experiments.

One of us (F. D.) is a member of the Scientific Staff of the Medical Research Council: the other (G. D. G.) is the holder of a Halley-Stewart Research Scholarship, for which he wishes to make acknowledgments here.

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CLII. INTERACTION OF HALOGENACETATES AND SH COMPOUNDS.

THE REACTION OF HALOGENACETIC ACIDS WITH GLUTATHIONE AND CYSTEINE. THE MECHANISM OF IODOACETATE POISONING OF GLYOXALASE¹.

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(Received July 3rd, 1933.)

SINCE Lundsgaard [1930] drew attention to the remarkably toxic nature of certain of the halogenacetic acids and their rôle as powerful and selective inhibitors of lactic acid formation and of alcoholic fermentation, these compounds have assumed great importance in physiology, and the numerous investigations of fundamental value to the theories of muscular contraction, tissue glycolysis and fermentation which have resulted show the usefulness of these labile halogen compounds as reagents in the study of intermediary tissue metabolism. But in spite of the many investigations, in no one case has the mechanism of their action been explicable in any clearly defined way, though various suggestions, such as those of Waldschmidt-Leitz and Schäffner [1932] and of Bersin [1932], have been advanced from time to time.

In view of the importance of the iodoacetate reaction, it was decided to investigate what appeared to be one of the simplest physiological transformations known at the time to be inhibited by iodoacetic acid, namely the conversion of methylglyoxal into lactic acid: a type of oxido-reduction of great interest and perhaps one directly concerned in glycolysis. Indeed Dudley [1931], who discovered the inhibitory action of iodoacetic acid on this keto-aldehyde mutase system, considered that this might well be the mode of action of the halogenacetic acids on the glycolytic process. It is true that Lohmann [1931], shortly after, found that in his glyoxalase preparations a much higher concentration of iodoacetate was needed to stop glyoxalase action than was required to check the formation of lactic acid by the Meyerhof muscle extract; he therefore considered that it was unlikely that the inhibition of muscle glycolysis could be due to poisoning of the glyoxalase system.

Nevertheless this point could not be said to be definitely cleared up, and during an attempt to investigate it further the work to be described in the present paper, which sheds some light on the mechanism of iodoacetate poisoning of glyoxalase, arose.

In view of the possibility that the method of preparation of the extracts might affect the concentration of iodoacetate necessary, the behaviour of

¹ A preliminary account of part of the work presented here appeared in a letter to *Nature* in January last [Dickens, 1933]. Owing to the author's change of laboratory, the completion of this work and its publication have been somewhat delayed.

dialysed and undialysed extracts was being investigated when a further publication from Lohmann appeared, containing the important discovery that reduced glutathione could act as co-enzyme to glyoxalase in converting synthetic methylglyoxal into lactic acid. Liver extract, from which the bulk of the proteins had been precipitated by an acetate buffer, lost nearly all its glyoxalase activity on dialysis, and this was found to be completely restored by addition of minimum quantities of reduced glutathione to the dialysed extract [Lohmann, 1932]. This discovery of Lohmann suggested as an obvious possibility that the action of iodoacetate might be upon the sulphydryl compounds in the extract, leading to destruction of glyoxalase activity by destruction of the essential co-enzyme. From his earlier paper it is evident that Lohmann [1931] did not favour the view that iodoacetate acts upon the co-enzyme systems, a suggestion put forward, certainly with little direct evidence, by Barrenscheen and Braun [1931]: on the contrary Lohmann believed that the action of iodoacetic acid was to destroy the enzyme itself.

In the present paper it is shown that (I) under physiological conditions of p_H and temperature, the halogenacetic acids readily react with sulphydryl compounds, in particular with glutathione and cysteine, forming the corresponding thio-ethers and hydrogen halide, and (II) that this action provides an adequate explanation for the destruction of glyoxalase activity by halogenacetic acids.

I. *The interaction of glutathione and cysteine with halogenacetic acids.*

Evidence of the reaction between iodoacetic acid and glutathione was first obtained manometrically. Glutathione, prepared by Pirie's [1930] method and purified through the cadmium salt [Voegtlin, Johnson and Rosenthal, 1931] was dissolved in glass-distilled water containing 0.031 *M* NaHCO_3 , the solutions

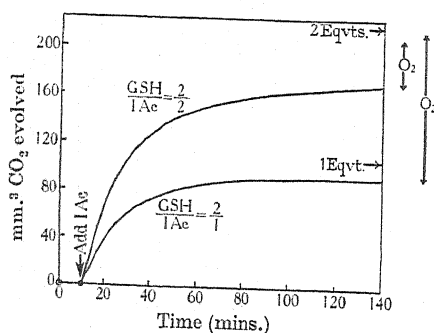


Fig. 1.

Fig. 1. *Reaction of iodoacetate with glutathione.* The oxygen uptake after 140 mins. (vessels filled with O_2 + 5% CO_2 and trace CuSO_4 added) is shown by the heavy lines on the right: the units are the same as for the CO_2 -liberation, and the sum of the latter with the O_2 -uptake of the unchanged GSH is near the theoretical for 2 equivalents GSH.

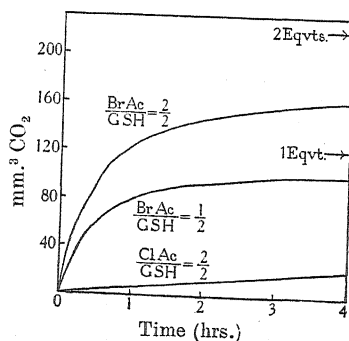


Fig. 2.

Fig. 2. *Reaction of bromoacetate and chloroacetate with glutathione.*

having been previously saturated with a gas mixture of nitrogen with 5% CO_2 . Suitable quantities (3 cc.) of the approximately *M*/300 solution of glutathione were measured into the Warburg manometer vessels, which were provided with side-bulbs containing the *N*/20 solution of the sodium salts of the halogenacetic acids (0.1 or 0.2 cc.): the latter solutions also contained a similar excess of

NaHCO_3 to that present in the glutathione solution in the main part of the vessel. After filling with nitrogen containing 5 % CO_2 , the vessels, attached to their manometers, were put into the thermostat at 37.5° and shaking was begun. When temperature equilibrium was attained the contents were mixed by tipping the vessel and then replacing in the thermostat. CO_2 evolution immediately began and its course was followed by the pressure readings (Figs. 1 and 2).

Table I.

37.5° ; $[\text{Na}^+] = 0.032$; $\text{N}_2 + 5\% \text{CO}_2$; p_{H} approx. 7.4.

Iodoacetate and glutathione:

$a = 3.15 \times 10^{-3}$, $b = 1.61 \times 10^{-3}$, vol. = 3.1 cc.

t mins.	x (mm. ³ CO_2)	x $M \times 10^{-3}$	k
10	38.5	0.55	14.5
20	61	0.88	15.1
30	72.5	1.05	14.1
40	81	1.17	13.6
Mean			14.3

$a = 3.05 \times 10^{-3}$, $b = 3.12 \times 10^{-3}$, vol. = 3.2 cc.

10	69.5	0.97	14.5
20	108	1.50	17.5
30	126	1.76	15.5
40	140	1.95	14.6
Mean			15.5

Bromoacetate and glutathione:

$a = b = 3.13 \times 10^{-3}$, vol. = 3.2 cc.

10	48.5	0.67	8.7
20	75.5	1.05	8.1
30	95	1.32	7.8
40	107	1.49	7.3
Mean			8.0

$a = 3.24 \times 10^{-3}$, $b = 1.61 \times 10^{-3}$, vol. = 3.1 cc.

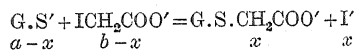
30	58.5	0.84	8.9
40	70.5	1.02	9.5
50	76	1.10	9.0
60	81	1.17	8.7
Mean			9.0

Chloroacetate and glutathione:

$a = b = 3.13 \times 10^{-3}$, vol. = 3.2 cc.

120	12.5	0.175	0.16
180	17.5	0.244	0.15
240	21.5	0.300	0.14
270	23	0.320	0.14
Mean			0.15

Table I shows that the reaction follows the bimolecular law and may be represented as follows:

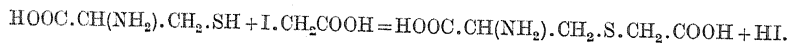


or similarly for the other monohalogenacetic acids. Similar reactions, *e.g.* of thiolacetic acid and halogenacetic acids, are well known [Klason, 1877; Klason and Carlson, 1906], but they have been surprisingly little studied by physical chemists. Quite recently, however, Hellström [1931] has studied the example (thiolacetic acid) mentioned from the point of view of Brönsted's theory and finds that the reaction follows the bimolecular law, though the velocity constant depends on the

nature and concentration of the cations present, being, when $[\text{Na}^+] = 0.0375$ and at 25° , of the order: 150, 70, 0.70 (uncorr.) for iodo-, bromo-, chloro-acetic acids respectively. This order is very similar to that shown in the present experiments, the constants with thiolacetic acid being however about 10 times as large, despite the lower temperature used by Hellström. This series is also in agreement with the physiological action, that is it corresponds with the order of activity of the three halogen-acids in checking glycolysis or fermentation [Lundsgaard, 1930; Lohmann, 1931; Cayrol, 1931].

Whilst the above course follows the bimolecular type sufficiently accurately, it was thought necessary to isolate the products of the reaction. With glutathione the purification of the condensation compound presented difficulties, and the reaction was worked out with cysteine, where the greater stability makes the separation easier.

For this purpose cysteine hydrochloride (0.16 g. = 1 milli-equiv.) was neutralised with $N/5 \text{ Ba(OH)}_2$ (5.5 cc.) and a neutral solution of iodoacetic acid (0.19 g. = 1 milli-equiv.), dissolved in 5.3 cc. $N/5 \text{ Ba(OH)}_2$ was added while N_2 was passed through the solutions. The reaction mixture at once became acid, and $N/5 \text{ Ba(OH)}_2$, in all a further 5.0 cc. (1 milli-equiv.), was added to keep the reaction mixture neutral; after addition of alcohol (15 cc.) the mixture was allowed to stand overnight. In the morning a further 30 cc. absolute alcohol was added and the white precipitate of Ba salts, including some BaCl_2 , was collected, after half an hour, in the centrifuge: the alcoholic supernatant liquid contained barium iodide. The precipitate of Ba salts was dissolved in water (3 cc.), and H_2SO_4 (ca. 10 cc. $N/10$) was cautiously added to give complete precipitation of the Ba as BaSO_4 ; the latter was removed and the clear colourless solution evaporated *in vacuo* over P_2O_5 . A white crystalline residue began to separate when the volume was reduced to a few cc., and the whole contents of the dish crystallised overnight: traces of a yellow impurity and of HCl were removed by washing with a little ice-cold alcohol. Yield 0.15 g. = 85 % of the theoretical; colourless clusters of fine needles, M.P. 84° decomp. (uncorr.). For micro-analysis (Schoeller) the crystals were dried in the air without further purification. Found: C, 33.14; H, 5.16; N, 7.10; S, 17.69 %. $\text{C}_5\text{H}_9\text{O}_4\text{NS}$ requires: C, 33.51; H, 5.06; N, 7.81; S, 17.69 %. The course of the reaction is therefore as follows:



The thio-ether is only slightly soluble in cold water or alcohol, more soluble in hot water and freely soluble in dilute aqueous ammonia. The aqueous solution is acid in reaction.

When the same method of isolation was tried with glutathione the recovery and purity of the product were not so satisfactory. Neutral solutions ($\frac{1}{3}$ milli-equiv.) of Ba salts of glutathione and iodoacetic acid were mixed, and in all 1.55 cc. $N/5 \text{ Ba(OH)}_2$ (calc. 1.66 cc.) were required to keep the mixture neutral. The alcoholic precipitate of Ba salts was freed from Ba by H_2SO_4 (6 cc. $N/10$). Evaporation of the filtrate *in vacuo* over P_2O_5 gave a clear sticky residue which changed to a feathery precipitate when rubbed with alcohol. The precipitate was collected and dried in a vacuum desiccator overnight. Micro-analysis (Schoeller) showed that the substance is probably the not quite pure thio-ether containing 1 mol. H_2O . (Found: C, 38.4; H, 6.0; N, 9.4; S, 7.9 %. $\text{C}_{12}\text{H}_{19}\text{O}_5\text{N}_3\text{S}$, H_2O requires: C, 37.6; H, 5.5; N, 10.9; S, 8.3 %.) Further purification of the thio-ether was not carried out: it was freely soluble in cold water, giving a solution with an acid reaction from which the substance was reprecipitated by addition of cold alcohol: the nitroprusside reaction was negative.

These experiments leave little doubt that the reaction is perfectly analogous to that with cysteine, where the condensation product is more readily isolated in the pure state.

Immediately following the publication of the preliminary report of these experiments [Dickens, 1933] the author learned in a personal communication from Dr Rapkine that he had, quite independently, studied the reaction of cysteine and iodoacetic acid: these results have now been published [Rapkine, 1933] and agree in all respects with those set out in the author's preliminary account. In addition the rate of reaction was shown by Rapkine to increase with increasing temperature and alkalinity. The reaction products (thio-ethers) were not isolated in Rapkine's experiments. Quastel [1933] has also produced interesting evidence of physiological interaction of sulphydryl compounds and iodoacetate, and in fact the paper by Quastel and Wheatley [1932] was, unknown to the author, submitted for publication before the appearance of his preliminary account. It may be mentioned here that one of the earliest recorded examples of the biological action of halogen compounds on thiol compounds appears to be that of Thunberg [1911].

II. *The inhibition of glyoxalase by iodoacetate.*

The source of glyoxalase in all experiments was rat-liver. For the preparation of the extracts the freshly removed liver was minced in a micro-mincer, ground with sand and extracted twice by thoroughly grinding with its own weight of water and centrifuging: the two extracts were then combined. For removal of protein, acetate buffer ($\frac{1}{10}$ vol. $M/3$, p_H 4) was used as in Lohmann's [1932] extracts, followed by centrifuging. $NaHCO_3$ (usually $\frac{1}{10}$ vol. 1.3 %) was added and the solution was dialysed in collodion sacs against distilled water. The water in the 1st and 2nd dialysates invariably showed a positive nitroprusside reaction: sometimes on dialysis a further slight precipitate formed, and was removed by centrifuging. In some extracts KCl (0.9 %) was used instead of water for the preliminary extraction, without any noticeable difference in the extract.

For the estimation of glyoxalase activity, chemically controlled manometric experiments were used, in which 1-2 cc. liver extract, usually diluted with an equal volume of water, were measured into the Warburg manometer vessels together with a volume of 1.3 % $NaHCO_3$ usually $\frac{1}{3}$ to $\frac{1}{2}$ the volume of the original extract, and the vessel was then filled with a gas mixture of 5 % CO_2 in N_2 . The temperature for the measurements with extracts was always 30°. The p_H was controlled by bicarbonate estimations, and the CO_2 -retention by acidification: with crude extracts the latter was about 20 %, but with deproteinised and dialysed extracts on the other hand the retention correction was small (about 1.5 %). Lohmann has shown that the evolution of CO_2 is an accurate measure of the lactic acid formed when suitable conditions are observed. In view of the possibility that this equivalence might be upset by the presence of iodoacetic acid, this point was controlled in selected experiments by determination of the lactic acid formed, by precipitation of proteins with the Schenk reagent and analysis of the filtrate, freed from mercury by H_2S , by Clausen's method, using Friedemann and Kendall's [1929] modification. Blanks on the extracts and methylglyoxal solutions used were invariably made, and the amounts of lactic acid found were corrected for these blank values. On the whole the agreement of manometric and chemical estimations was satisfactory, and usually 90-98 % of the manometric readings were given by the Clausen analysis. Only when very high concentrations ($M/100$) of iodoacetate were

used did there appear to be some tendency for the chemical values to be lower compared with the manometric ones, but these experiments were not important for the present purpose, as such high concentrations of iodoacetate were seldom used.

The methylglyoxal solutions used were obtained by the method of Neuberg *et al.* [1917] from the distillation of dihydroxyacetone with dilute sulphuric acid. The distillate (50 cc. from 1 g. dihydroxyacetone) was neutralised to phenolphthalein with $N/10$ NaOH before use and NaHCO_3 (1.3 %) to give a similar concentration to that in the extract was then added: 0.1 to 0.3 cc. of the distillate so treated was used for each experiment, being usually measured into the side bulb of the manometer vessels.

Glutathione was made by Pirie's [1930] method from yeast, was crystallised from water and then purified through the cadmium salt [Voegtlin, Johnson and Rosenthal, 1931], afterwards being again crystallised from water by evaporation over H_2SO_4 at room temperature. Dissolved in glass-distilled water it was sufficiently stable (5.0 mg. in 3 cc. $M/75$ phosphate or in bicarbonate buffer at p_{H} 7.4 took up about 4 mm.³ O_2 at 38° in 60 mins.), but on the addition of CuSO_4 ($5 \times 10^{-5} M$) the oxygen uptake was 98.5 % of the theoretical and was complete in 60 minutes. (In confirmation of Voegtlin, Johnson and Rosenthal's finding it may be mentioned that 6 times this amount of Fe, in the form of thrice recrystallised ferric ammonium sulphate, was almost without effect on the O_2 uptake in phosphate buffer.)

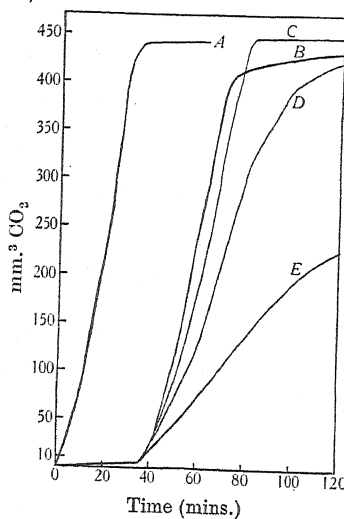


Fig. 3. Action of iodoacetate on undialysed liver extract.

A. Control, methylglyoxal added at 0 mins.
B. Control, methylglyoxal added at 35 mins.
C, D and E; Iodoacetate added at 0 mins. and methylglyoxal at 35 mins. Concentration of ICH_2COOH : C, $5 \times 10^{-4} M$; D, $2.4 \times 10^{-3} M$; E, $1 \times 10^{-2} M$. Chemical estimation of lactic acid formed: A, 1.72; D, 1.53; E, 0.70 mg. (96, 90 and 80 % respectively of that calculated from pressure changes).

The iodoacetic acid was a pure specimen kindly given me by Dr Lundsgaard. Bromoacetic acid was purified by recrystallising from ligroin, and chloroacetic acid by redistillation *in vacuo*. These acids were dissolved in glass-distilled water and neutralised with NaHCO_3 . All glass-ware was cleaned with chromic acid

and well rinsed with glass-distilled water before drying. Naturally, the liver extracts were not metal-free, but the use of these precautions prevented loss of part of the glutathione by oxidation before the experiment was begun.

Crude liver extract. A portion of the acetate-precipitated liver extract was taken before dialysis, or in another experiment after only half an hour's dialysis to remove the greater part of the acetate. The CO_2 -retention correction of the former was 20 % and of the latter only 6 %. Bicarbonate was added and $\text{N}_2 + 5\% \text{CO}_2$ passed, when the p_{H} was 7.4. These extracts were strongly active without added GSH; relatively high concentrations of iodoacetic acid were needed to inactivate them. From Fig. 3 it will be seen that the activity of the crude extract is not impaired by incubation. Iodoacetate in $N/2000$ concentration had no action; $N/420$ caused a perceptible though slight retardation of lactic acid formation whilst the $N/100$ solution inhibited to about 50 %. The chemical estimations showed that the manometric estimations fairly represented the lactic acid formed with the two lower concentrations (96 and 90 % of the manometric readings corresponded to the amounts of lactic acid found by Clausen determination); with the concentrated solution the discrepancy was greater. The experiment shows that a high concentration of iodoacetate is necessary to inactivate the undialysed extract.

Dialysed liver extracts. Fig. 4 shows the reactivation of an extract dialysed for 3 hours by the addition of glutathione after the extract had been inactivated by incubating for 70 mins. with $N/100$ and $N/420$ iodoacetate. Recovery of activity was complete and instantaneous on the addition of GSH equivalent only to $\frac{1}{20}$ of the amount of iodoacetate added. This effect is still clearly shown by the extract after prolonged dialysis has reduced almost to zero its power to form lactic acid without any addition other than methylglyoxal. Fig. 5 shows the behaviour of an extract dialysed for 12 hours and the amounts of glutathione necessary to restore its activity. This extract was then shaken for 40 mins. in the thermostat with the addition of methylglyoxal to all vessels, and to some of iodoacetate also, in suitable concentrations. Glutathione (0.01 mg.) was then added from the side-bulbs attached to the vessels, when as Fig. 6 shows, reactivation was complete with those vessels which had 10^{-4} and $10^{-3} M$ iodoacetate (the latter is a 30-fold excess over the amount of glutathione used). On the other hand in the 300-fold excess present in the case of $M/100$ iodoacetate, this small amount of glutathione was unable to bring about complete reactivation, and the lactic acid formation as shown both by manometric and chemical estimation fell to about 60 % of the control without iodoacetate. The explanation of the phenomenon of reactivation in the presence of an excess of the inhibitor, which has been repeatedly confirmed, might lie either in a destruction of iodoacetate by incubation with liver extract, for which there is no direct evidence, or it is more probably due to a much more rapid reaction of GSH with the glyoxalase system than the reaction of GSH with iodoacetic acid. The reaction with the enzyme system is certainly rapid, in view of the immediate recovery of activity resulting in an instantaneous and vigorous formation of lactic acid on adding GSH.

The behaviour of the dialysed extract is very different from that of the cruder, undialysed preparations. Fig. 7 shows an experiment with the same extract as that used for the experiment shown in Fig. 4, but after a further 5 hours' dialysis (8 hours in all) the glyoxalase action, without addition other than methylglyoxal, is now reduced to a low level, though the spontaneous increase of activity with time [*cf.* Lohmann, 1932] was rather unusually pronounced with this extract, as is shown by the upward curvature of the controls.

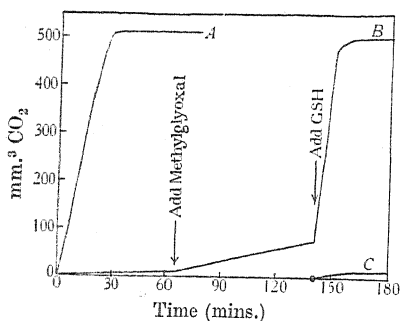


Fig. 4.

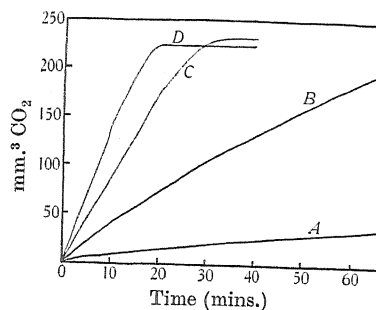


Fig. 5.

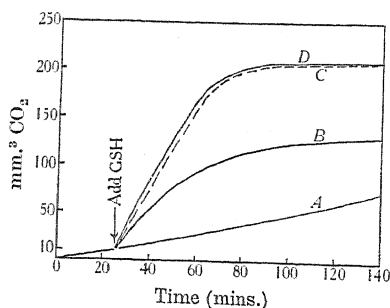


Fig. 6.

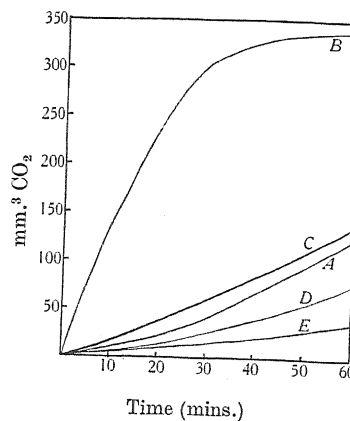


Fig. 7.

Fig. 4. Liver extract dialysed for three hours. Reactivation by GSH after inactivation by ICH_2COOH .

A. Control, methylglyoxal added at 0 mins.

B. (a) 2.4 mg. and (b) 0.47 mg. ICH_2COOH added at 0 mins. Methylglyoxal added at 65 mins., and 0.2 mg. GSH added at 140 mins.

C. Addition of 0.2 mg. GSH to 2.4 mg. ICH_2COOH .

Fig. 5. Amount of GSH needed to activate extract dialysed for 12 hours.

A. Control without added GSH. B, 0.01 mg.; C, 0.025 mg.; D, 0.05 mg. GSH.

Fig. 6. Reactivation of dialysed extract by GSH after ICH_2COOH .

The same extract as in Fig. 5 was put in the bath at 30° 15 mins. before the experiment began, with the addition of methylglyoxal and iodoacetate.

A. Control without added GSH.

B. ICH_2COOH , 10^{-2} M. GSH (0.01 mg.) added after 40 mins. treatment at 30° with ICH_2COOH .

C. ICH_2COOH , 10^{-3} M. Otherwise as B.

D. ICH_2COOH , 10^{-4} M. Otherwise as B.

Chemical controls. B, 0.48 mg. (91 %); C, 0.79 mg. (95 %); D, 0.785 mg. (95 % of manometric).

Fig. 7. Extract dialysed for eight hours.

A. Control without added GSH or ICH_2COOH .

To B, C, D and E was added 0.05 mg. GSH (1.2×10^{-4} M), and to C, D and E iodoacetate also, 20 mins. before experiment began. Methylglyoxal was added at 0 mins.

B. Control without ICH_2COOH ; C, ICH_2COOH , 1.2×10^{-4} M; D, 3×10^{-4} M; E, 2.5×10^{-3} M ICH_2COOH .

This extract was strongly activated by addition of glutathione in $1.2 \times 10^{-4} M$ concentration, and an exactly equivalent concentration of iodoacetic acid reduced the lactic acid formation to the level of the control without added GSH. The dialysed extract is therefore more than one hundred times as sensitive to iodoacetate as the cruder extracts, and the amount of iodoacetate required to bring about inactivation is almost exactly equivalent to the amount of glutathione added.

Intact tissues. The behaviour of slices of intact liver, studied by Warburg's method, by suspending in 2 cc. bicarbonate-Ringer solution at 38° and p_H 7.4, in an atmosphere of $N_2 + 5\% CO_2$, much more closely resembles that of the highly purified extract than it does that of the crude glyoxalase preparations. Two experiments are reproduced in Figs. 8 and 9. In the former equal weighed

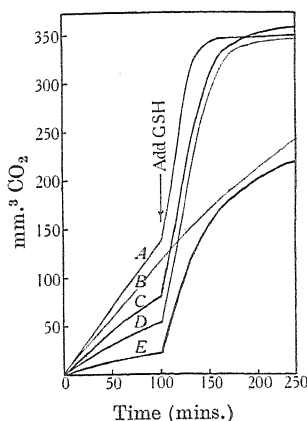


Fig. 8.

Fig. 8. Rat-liver slices.

Each vessel contained 50 mg. moist weight; all were put into the bath 60 mins. before first reading at 0 mins., when methylglyoxal was added.

A and B. Controls without ICH_2COOH ; C, $ICH_2COOH = 1.5 \times 10^{-4} M$; D, $4.8 \times 10^{-4} M$; E, $2.4 \times 10^{-3} M$ ICH_2COOH . 0.1 mg. GSH added to all except B at 100 mins.

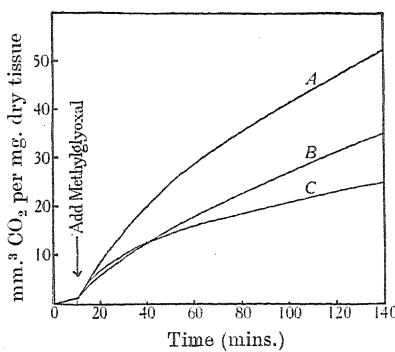


Fig. 9.

Fig. 9. Rat-liver slices.

A. Control without ICH_2COOH .

B. Time incubated 20 mins. with $1.5 \times 10^{-4} M$ ICH_2COOH before adding methylglyoxal.

C. As B but with $5 \times 10^{-4} M$ ICH_2COOH .

portions (50 mg.) of the slightly rinsed slices were placed in the vessels and to some of these iodoacetic acid in concentration varying from 1.5×10^{-4} to $2.4 \times 10^{-3} M$ was added. The slices were shaken in the manometer vessels in the thermostat for 70 mins. before adding the methylglyoxal solution (0.2 cc.) from the side-bulbs attached to the vessels. Definite inhibition of glyoxalase action was shown in the presence of even $1.5 \times 10^{-4} M$ iodoacetate, whilst with 3 times this concentration the activity was only about 40 % of the controls. The activation on adding glutathione (0.1 mg.) to the contents of the vessels was very marked, though with the highest concentration of iodoacetate used ($2.4 \times 10^{-3} M$) the conversion of the methylglyoxal was only partially complete (Fig. 8).

It was found that this long preliminary treatment of the liver slices with iodoacetate is unnecessary. In the experiment represented in Fig. 9 the conditions were the same, but the methylglyoxal (not the same solution as in Fig. 6) was added to the tissues 10 minutes after the first reading (in all 20 minutes after adding the iodoacetate and placing in the bath). The curves show that there is a definite inhibition of glyoxalase activity in the intact tissue slices by $1.5 \times 10^{-4} M$ iodoacetate, even after this short incubation time.

SUMMARY.

1. When neutral solutions of sodium iodoacetate and glutathione are mixed a reaction occurs which follows the bimolecular law and results in the elimination of iodide and the formation of a thio-ether. The behaviour of bromoacetate is similar but less vigorous, whilst that of chloroacetate is much less so; the respective velocity constants for the reaction at 38° are of the relative order, I:Br:Cl=15:9:0.15. With cysteine a similar reaction occurs, and the pure reaction product has been prepared in good yield under conditions of reaction and temperature not very different from the physiological.
2. The iodoacetate-inhibition of glyoxalase activity is reversed completely by addition of glutathione to the inactivated extract. It is therefore the coenzyme of glyoxalase that is inactivated by iodoacetate, the enzyme itself remaining undamaged. The concentration of iodoacetate necessary to inhibit glyoxalase preparations depends on the method of preparation of the extracts. With crude undialysed extracts high concentrations of iodoacetate ($N/100$) are necessary, whilst with the preparations subjected to thorough dialysis the amount of iodoacetate is nearly equivalent to the content of added glutathione, thus iodoacetate in about $10^{-4} M$ concentration or less may be sufficient to stop lactic acid formation from synthetic methylglyoxal in the dialysed extracts. This is of the same order of concentration as that found to inhibit the glyoxalase action of tissue slices, prepared and studied by Warburg's method. Hence it is once more shown that quantitative conclusions drawn from the behaviour of extracts may be misleading when applied to the intact tissues. The concentration of iodoacetate needed to check the conversion of synthetic methylglyoxal into lactic acid by intact tissues is of the same order as that [Krebs, 1931] required to stop the conversion of glucose into lactic acid by tissues.

Part of this work was carried out while the author was a member of the Scientific Staff of the Medical Research Council, and it has been completed at the Cancer Research Institute, North of England Council of the British Empire Cancer Campaign, Newcastle-on-Tyne. To both of these bodies he wishes to express his thanks. The United Yeast Company, Ltd., kindly provided facilities for the supply of yeast used for the preparation of glutathione.

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CLIII. THE APPROXIMATE DETERMINATION OF SPERMINE IN SINGLE HUMAN ORGANS.

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(Received May 20th, 1933.)

IN the writer's published work [Harrison, 1931] on the distribution of spermine in human tissues it was necessary to pool the smaller organs from a number of subjects in order to make a single estimation by the steam-distillation method. As a development of the subsequent study of Barberio's picric acid test for semen [Harrison, 1932] the method for estimating spermine in organs has been modified, so that approximate determinations of the amount in a single prostate, or in a pair of testes or in a single sample of human semen can be undertaken. The investigation is an extension of the work previously published, in the hope of throwing some light on the significance of the relatively large quantities of spermine in the average human prostate.

The modified method.

In brief, spermine and other substances are extracted from the dried material by trichloroacetic acid. An aliquot part of the cleared extract is precipitated with picric acid. The crude spermine picrate is converted into the hydrochloride, and then into the characteristic phosphate which is insoluble in 25 % alcohol. The spermine phosphate is separated, washed, dried and weighed.

The details are as follows. After mincing and weighing, the organ is heated on the boiling water-bath for several hours, absolute alcohol being added at intervals to hasten the drying process. It is then stored in a vacuum-desiccator as long as is convenient. After weighing, the dried organ is extracted with ether for 2 to 6 hours in a Soxhlet apparatus (when very fatty it is more satisfactory to weigh the organ after ether extraction). The residue is transferred quantitatively from the Soxhlet thimble into a glass-stoppered measuring cylinder of suitable capacity. 2.5 % trichloroacetic acid is added to a total of 10 volumes; i.e. each g. of dried (or of dried and ether-extracted) material is made up to a total of 10.0 cc. by addition of the acid. The mixture is thoroughly shaken at intervals, extraction being allowed to continue for 2 hours. Any contraction in volume is made good by addition of more acid, and, after mixing, the extract is decanted and cleared as much as possible by centrifuging or filtering or both. As a rule the final fluid is clear or only slightly opalescent. Its volume is measured, and an equal volume of saturated aqueous picric acid is added. The mixture is heated on the boiling water-bath till it is clear or only slightly opalescent. The flame is turned out and cooling allowed to proceed slowly to room temperature. (This makes the subsequent separation of the picrate less difficult.) After standing for a few hours (conveniently overnight), the picrate precipitate is separated by decanting and centrifuging (filtration is generally less satisfactory), and is roughly dried in air (e.g. in the incubator at 37° overnight). It is then dissolved in the minimum of boiling acid alcohol (concentrated HCl 7 cc., water 43 cc., alcohol to 200 cc.), and thrown into at least 10 volumes of acetone. The precipitate of crude spermine hydrochloride is filtered off, most conveniently through a Soxhlet thimble placed in a conical funnel which is mounted in a filter-flask, so that slight suction can be applied at the end of the process. After washing with acetone until free from picric acid, the funnel is transferred without delay to a suitable boiling-tube with side-arm. The precipitate

is dissolved in the minimum of hot water, a few cc. being added at a time followed by gentle suction. Phenol red is added as indicator, and then drop by drop a concentrated aqueous solution of diammonium hydrogen phosphate until a p_H of 7.0 to 7.2 is attained. After adding 1/3 vol. of absolute alcohol, the mixture is allowed to stand overnight at room temperature for the crystals of spermine phosphate to separate. The crystals are finally filtered off, washed with 25 % and then with absolute alcohol, dried and weighed. The following is an example of the calculation:

Fresh weight of minced prostate, 22.85 g.

Dry weight of same, 4.52 g.

Made up to total volume of 45.2 cc. with 2.5 % trichloroacetic acid.

Volume of filtrate, 30.5 cc., which corresponds to 3.05 g. of dried prostate.

Weight of dry spermine phosphate, 9.5 mg.

Therefore yield of spermine (as phosphate) = $\frac{9.5 \times 100}{3.05} = 311$ mg. per 100 g. dry prostate,

or $\frac{9.5 \times 100}{3.05} \times \frac{4.52}{22.85} = 61.5$ mg. per 100 g. fresh prostate.

Method for human semen.

A measured volume of semen is thrown into at least 5 volumes of absolute alcohol, mixed well and allowed to stand several hours (*e.g.* overnight). The precipitate of protein and spermine phosphate and other substances is filtered off with suction, washed with absolute alcohol and dried in air. The precipitate from each cc. of semen is made up to 5 cc. with 2.5 % trichloroacetic acid, and treated subsequently exactly as described above.

Notes on method.

The organ must be dried and (in most cases at any rate) extracted with ether. If the fresh organ is extracted directly with trichloroacetic acid, the extract contains fat and other substances in such a fine state of division that it cannot be cleared satisfactorily by centrifuging or filtering.

The crude spermine picrate generally comes out so finely divided that it is difficult or impossible to separate it by filtration. By redissolving with heat and slow cooling, followed by centrifuging, this difficulty is largely eliminated.

By the method above described the weighing of the picrate [see Harrison, 1931] can be avoided—an important point when dealing with such small quantities.

If the yield of spermine phosphate is relatively large, or if it is grossly pigmented, it is better to complete the precipitation by adding an equal volume of alcohol, then to redissolve the precipitate in boiling water, decolorise with charcoal and filter hot; phenol red is added to the filtrate, the reaction is adjusted if necessary, and 1/3 vol. of absolute alcohol is added as previously described [Harrison, 1931].

Several experiments were made on the time required for extraction by trichloroacetic acid. There was no significant difference in the yields when half the gland was extracted for 2, and the other half for 24 hours.

For the reason given below, the present method is not so accurate as the steam-distillation method. The latter is therefore to be preferred when working with large organs or with large numbers of pooled small organs. The present method is useful for single small organs, or as a preliminary test to discover roughly how much spermine an organ or other material contains.

DISCUSSION OF RESULTS.

The organs were all obtained *post mortem*; the cause of death is indicated in the Tables. To outward appearance none of the organs analysed was diseased; those cases in which abnormalities of the particular organs would be expected from the nature of the cause of death were excluded. The results have been arranged according to the subjects' ages. Some of the pancreases obtained for analysis were incomplete, portions being required for other examinations. When

examined microscopically the crude spermine phosphate finally obtained was seen, in the case of the prostates and human semen, to consist almost entirely of the typical lenticular crystals. The final material from testes contained a little amorphous matter. The crude phosphate from pancreas contained quite an appreciable amount of amorphous matter; the yields reported for spermine

Table I. *Prostates.*

Patient	Age years	Spermine (as phosphate) mg. per 100 g.		Weight of prostate, g.		Cause of death
		Dry gland	Fresh gland	Dried	Fresh	
W. S.	8	None detected		0.19	0.96	Cerebrospinal fever
L. W.	15	47	—	1.03	—	Malignant endocarditis
C. M. T.	19	188	—	1.34	—	Septic endocarditis
G. T.	31	427	—	1.47	—	Lymphadenoma
G. G.	31	346	66	3.93	20.7	Subphrenic abscess: septic meningitis
W. M.	35	326	—	2.20	—	Exophthalmic goitre
J. C.	38	625	117	2.40	13.3	Tuberculous bronchopneumonia
H. P.	39	1219	266	5.24	24.1	Pontine haemorrhage
F. B.	39	227	45	4.34	21.9	Addison's disease
R. T.	41	628	125	2.25	11.4	Carcinoma of pancreas
J. S.	42	86	15.6	2.46	13.5	Hodgkin's disease
E. R.	47	311	62	4.52	22.9	New growth and abscess lung
F. B.	49	1410	—	2.80	—	Gastric ulcer: broncho-pneumonia
R. T.	50	158	26	3.05	18.8	Familial jaundice: peritonitis
W. E.	55	663	—	3.00	—	Gastric ulcer
W. W.	57	1293	—	2.32	—	Gastrectomy: peritonitis
W. Wd.	59	506	98	6.07	31.3	Fractured skull
R. M.	60	965	—	3.04	—	Contracted kidney: uraemia
W. C.	60	989	182	4.06	22.1	Emphysema; cardiac failure
J. B.	61	1358	255	2.10	11.2	Rheumatic carditis
G. P.	65	557	145	13.72	52.7	Contracted kidney: uraemia
W. B.	66	695	131	3.26	17.4	Perforated gastric ulcer
W. N.	66	22	5.6	3.09	12.0	Renal tuberculosis and perinephric abscess
P. S.	66	930	173	4.69	25.2	Abdominal neoplasm
J. M.	67	387	82	2.16	10.2	Myocarditis
J. G.	72	302	61	2.75	13.6	Duodenal ulcer: arteriosclerosis
Average*:		582.6	109.1			

* Excluding W. S., age 8.

Table II. *Testes.*

Patient	Age years	Spermine (as phosphate) mg. per 100 g.		Weight of pairs of testes, g.		Cause of death
		Dry gland	Fresh gland	Dried	Fresh	
C. C.	32	102	16.0	6.24	39.9	Arthritis of hip: septicaemia
G. McC.	34	108	14.5	4.91	36.7	Chronic rheumatic carditis
C. S. C.	37	47	7.1	4.54	30.4	Chronic nephritis
C. H.	38	92	12.2	3.36	25.2	Carcinoma in lung
W. B.	42	23	3.5	4.20	27.3	Coronary thrombosis
H. B.	45	12	1.7	2.05	14.1	Phthisis
A. B.	46	58	9.8	4.11	24.4	Contracted kidney: uraemia
C. B.	50	71	12.0	5.36	31.7	Carcinoma of bladder
C. H.	57	70	11.6	6.39	38.3	Carcinoma of pancreas
T. B.	61	71	11.0	3.15	20.4	Peritonitis
H. S.	61	81	12.0	4.54	30.5	Pelvic neoplasm
R. C.	62	52	9.3	5.62	31.3	HCl poisoning
C. A.	65	65	10.0	3.83	24.7	Carcinoma of liver
W. B.	66	29	4.4	2.64	17.7	Perforated gastric ulcer
Average:		63	9.7			

Table III. *Pancreases.*

Patient	Age years	Spermine (as phosphate) mg. per 100 g.		Weight of pancreas, g.		Cause of death
		Dry gland	Fresh gland	Dried	Fresh	
T. E.	16/365	Present		0.31	1.09	Umbilical sepsis: bronchopneumonia
F. G.	4	303	61	2.24	11.1	Diarrhoea and vomiting
W. S.	8	253	54.5	6.14	28.5	Cerebrospinal fever
W. McS.	19	201	43	16.81	78.9	Acute rheumatic carditis
E. R.	26	123	27	6.78	30.9	Mediastinal lymphosarcoma
H. B.	37	151	50	12.55	38.1	Peritonitis
J. C.	38	123	45	22.69	61.6	Tuberculous bronchopneumonia
J. S.	42	230	40	12.59	72.4	Hodgkin's disease
H. G.	50	254	46	8.36	45.9	Perforated gastric ulcer
F. S.	50	156	30	15.27	79.9	Lymphatic leucaemia
W. E.	55	210	—	14.65	—	Gastric ulcer
W. C.	60	159	16.3	3.89*	37.8	Emphysema; cardiac failure
J. B.	61	105	13.5	13.08*	102.0	Rheumatic carditis
G. P.	65	117	15.1	8.13*	62.9	Contracted kidney; uraemia
J. G.	72	239	50.8	14.03	66.0	Duodenal ulcer; arteriosclerosis
Average:		187.4	37.9			

* Weight of dried organ less ether-soluble matter.

Table IV. *Miscellaneous.*

Table IV. <i>Incumbitions.</i>							
Material	Age years	Spermine (as phosphate) mg. per 100 g.		Weight of material, g.		Cause of death	Amorphous impurity in spermine phosphate
		Dried	Fresh	Dried	Fresh		
Stomach	15	127	22	22.9*	129.9	Miliary tuberculosis	Little
	50	101	18	22.1	127.8	Suppurative cholangitis: carcinoma of common bile-duct	"
	63	79	14	24.0	138.9	Duodenal ulcer	"
Small intestine	15	167	20	43.8*	364.1	Miliary tuberculosis	Fair quantity
	47	141	19	58.0*	428.6	Gastric ulcer: peritonitis	Little
	48	129	17	19.2*	150.4	Pneumonia	"
Large intestine	15	34	4.6	33.2	247.0	Miliary tuberculosis	Fair quantity
	47	65	8.6	25.2*	191.0	Gastric ulcer: peritonitis	"
	48	182	24	57.6*	444.1	Pneumonia	Little
Spleen	40	76	16	57.0*	275.6	Cerebral abscess: empyema	"
	53	111	26	20.5*	88.0	—	"
	55	52	13	24.8*	100.5	—	"
	65	112	22	12.7*	65.3	Carcinoma of common bile-duct	"
Uterus	42	31	8	11.5	44.5	Carcinoma of colon: peritonitis	Fair quantity
	49	46	11	22.1	92.5	Mediastinal growth	"
	54	18	5	11.4	41.3	Lobar pneumonia	"
Pairs of ovaries	41	26	4	2.0	12.8	Suppurative perinephritis	Considerable
	54	11	2	1.1	4.4	Lobar pneumonia	"
Thyroid	49	20	5	2.0	7.8	Mediastinal growth	Fair quantity
	57	15	5	5.8	15.6	Duodenal ulcer	"
	65	7	2	3.5	13.8	Suppurative diverticulitis: gangrene of colon	"
		Date (1932)	mg./ 100 cc.		cc.		
Semen	38	26. ii	233	—	3.0	Same individual	
		4. iii	112	—	3.5		
		15. iii	268	—	3.3		
		29. iv	170	—	4.5		
		14. vi	160	—	3.2		
		21. vi	154	—	2.7		
	25	—	63	—	1.0	Healthy individual	

* Weight of dried organ less ether-soluble matter.

phosphate are therefore definitely too high, though probably not more than + 10 %. With some of the other organs reported in Table IV the proportion of amorphous matter was still greater (see last column of Table IV), but the figures at least indicate that in all these organs, spermine, though present, is not in excess of the amounts found generally elsewhere; they extend the contrast between the yields from prostates and other organs respectively, because several of the organs in Table IV had not been examined in the previous investigation.

The amorphous matter in the crude spermine phosphate finally separated was mainly protein or cleavage products of protein.

The average yield from prostates (Table I) in this series (109.1 mg. per 100 g. of 17 fresh prostates) is of the same order as the previous yield [Harrison, 1931] of 130 mg. per 100 g. from 1594 g. of pooled organs collected under alcohol. But the individual results vary enormously (5.6 to 266 mg.), and no correlation is apparent between yield and either the patient's age, or the size of the gland or the type of disease causing death. The range is so great as to make it unlikely that similar estimations in diseases of the prostate would be of any value. It would seem that the quantity of spermine in the prostate is fortuitous. Possibly any excess in the body is excreted by this gland. Certainly the results do not suggest that spermine has any particular functional significance in connection with the prostate or with reproduction.

It has been possible to make only a few determinations on normal human semen (Table IV). The results vary considerably; this is strikingly shown in the series of observations on one individual over a period of 4 months. Since the spermine of semen is probably mainly if not entirely derived from the prostate [Harrison, 1931] these findings are to be expected.

The spermine contents of 14 individual pairs of testes (1.7 to 16 mg., Table II) vary much less than those of prostates. They are of the same order as previously reported for pooled testes and are not above the amounts found generally in the organs of the body.

The results for 13 individual pancreases (Table III) vary from 13.5 to 61 mg. per 100 g. fresh gland, with a mean of 37.9 mg., as against the value of 16.1 mg. previously reported for 3361 g. of pooled pancreases. As explained above the present figures are a little too high in view of impurities.

SUMMARY.

1. A method is described for the approximate determination of spermine; this is useful in examining single small organs or a single sample of semen, and as a preliminary test to discover whether material contains significant amounts of spermine.

2. The spermine contents of individual normal prostates obtained *post mortem* vary enormously. Though the average yield is high, confirming previous work, the conclusion is drawn that the amount is fortuitous, possibly representing an excretion of excess from the body. The results throw no light on the possible functional significance of spermine.

3. The approximate spermine contents of a few more human organs are reported; they are of the same order as found in all organs other than the prostate.

The author is indebted to the Medical Research Council for a grant towards the expenses of this work.

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CLIV. THE RELATION OF LACTIC ACID AND ALANINE TO GLYCOGEN FORMATION.

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(Received July 1st, 1933.)

THE intermediary stages in the transformation of alanine into glucose are not clearly understood. The amino-acid may either undergo hydrolytic deamination yielding lactic acid, or be converted into pyruvic acid by oxidative deamination. Evidence that it is finally converted into sugar and the quantitative relationship which exists have been furnished by making use of the glycosuric organism. Thus Ringer and Lusk [1910] gave 20 g. of *dl*-alanine to a phlorrhizinised dog and observed its complete elimination as 20 g. of urinary glucose. Mandel and Lusk [1906] showed that *d*-lactic acid was completely converted into glucose in the diabetic organism and that 70 % of the *dl*-lactic acid could be so transformed. Ringer [1913] showed that pyruvic acid is convertible into glucose when administered to the glycosuric organism.

An alternative method of demonstrating the conversion of amino-acid into glucose, and one which can be shown in a normal organism, seems to be the measurement of liver- and muscle-glycogen following administration of the amino-acid. Neuberg and Langstein [1903] gave 20-30 g. of *dl*-alanine to starving rabbits and found from 1 to 2 g. of glycogen in the liver. Muscle-glycogen was not measured. They recovered from the urine of their animals an amount of lactic acid corresponding to 2 g. of zinc lactate. Regarding the formation of glycogen from lactic acid itself contradictory results have been obtained. Barrenscheen [1914] perfused the isolated livers of rabbits and dogs with sodium lactate and was unable to demonstrate glycogen formation. Abramson, Eggleton and Eggleton [1927], using dogs anaesthetised with ether and amytal, were unable to demonstrate glycogen synthesis in the liver following intravenous injection of sodium *dl*-lactate, although the injection of glucose under the same conditions yielded glycogen in the liver. Cori and Cori [1929] found that sodium lactate, when fed by mouth or injected subcutaneously, led to glycogen deposition in the liver.

Efforts to demonstrate an increase in liver-glycogen in normal animals are complicated by such important considerations as the obtaining of reliable controls and the use of operative procedures designed to prevent excessive excitation. Adult rabbits are not a good medium for work of this kind on account of the marked irregularity in the glycogen content of the tissues even when every precaution is taken to have the animals fed and otherwise treated alike. Young rabbits on the other hand are capable of furnishing reliable controls, especially when of the same litter, because of the uniformity of their liver- and muscle-glycogen content.

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EXPERIMENTAL.

Young rabbits of the same litter were used. Blood-sugar was determined by MacLean's method, blood from the carotid artery being taken. Glycogen was determined in liver and muscle in the following manner. The feet and head of the animal were held and the carotid artery on one side severed with a sharp razor. From the blood thus obtained appropriate amounts for the various estimations were immediately taken. The animal was killed by a sharp blow on the back of the head, extended and the abdomen rapidly opened. The liver was quickly removed, the gall bladder detached, the posterior lobe cut off, weighed and dropped into boiling 60 % potassium hydroxide (1 cc. to 1 g. tissue). This process, from the time of killing the animal until the addition to the potassium hydroxide, did not occupy more than 90 seconds. Meanwhile the posterior extremities were skinned and the anterior tibial and gastrocnemii muscles removed, weighed and dropped into boiling potassium hydroxide. The remainder of the liver was weighed and discarded. The tissue was allowed to hydrolyse in the potash for 3 hours, then 3 cc. of glacial acetic acid were added and the mixture filtered. Of the filtrate an aliquot part was taken into a hard glass centrifuge-tube and precipitated with absolute alcohol. After standing one-half hour the tube was centrifuged, the supernatant fluid drained off, 2 cc. of 2.2 % hydrochloric acid added, and the glycogen hydrolysed to glucose in a boiling water-bath. After cooling and neutralising with KOH the solution was made up to a known volume and filtered through a starch-free filter-paper. The glucose was estimated by the method of von Issekutz and von Both [1927] which depends on the reduction of potassium ferri-cyanide, and the quantities were so adjusted that amounts of glucose only up to 15 mg. could be estimated. This technique is particularly suitable for accurate glycogen determinations.

Blood- and urine-lactic acid estimations were made by the method of Friedemann, Cotonio and Shaffer. Blood- and urine-amino-acid estimations were made by the method of Folin.

Exp. 1. Ten young rabbits were taken. They were deprived of food for 24 hours before experiment. Five were used as controls and the other five were each given an intravenous injection of 2 g. of sodium *dl*-lactate. The animals were killed 3 hours after injection. The control animal and the corresponding injected animal are from the same litter in each case.

No.	Weight (g.)	Blood-sugar (mg./100 cc.)	Glycogen (%)		Liver- glycogen (mg.)	Blood- lactic acid (mg./100 cc.)
			Liver	Muscle		
<i>Controls:</i>						
1	1000	90	0.481	0.371	143	64
2	1100	58	0.513	0.584	163	44
3	850	45	0.292	0.110	63	33
4	900	45	0.643	0.592	195	44
5	850	51	0.726	0.362	163	24
<i>Injected animals (2 g. sodium dl-lactate intravenously):</i>						
6	1100	67	1.635	0.314	579	83
7	1100	79	1.512	0.450	553	96
8	1000	62	1.594	0.148	503	93
9	930	168	2.056	0.461	744	66
10	850	330	1.850	0.371	445	67

These results show an average increase in liver-glycogen of 400 %. There is no significant change in the glycogen content of muscle, nor in the blood-

sugar values. The average blood-lactic acid value rose from 41.8 mg./100 cc. in the control animals to 81 mg./100 cc. in the injected animals. The amounts of lactic acid in the urines of Nos. 7, 9 and 10 were measured and found to be 0.39 g., 0.113 g. and 0.196 g. This shows a variable excretion of from 5 to 20 % of the injected sodium lactate as lactic acid in the urine.

Exp. 2. Four animals from the same litter were taken and two of them given 2 g. of sodium *dl*-lactate intraperitoneally each. Before experiment, Nos. 1 and 3 were deprived of food for 12 hours, Nos. 2 and 4 for 24 hours, in order to show whether a greatly depleted liver-glycogen was a *sine qua non* for glycogen formation.

No.	Weight (g.)	Blood-sugar (mg./100 cc.)	Glycogen (%)		Liver- glycogen (mg.)	Blood- lactic acid (mg./100 cc.)
			Liver	Muscle		
<i>Controls:</i>						
1	1300	85	4.990	0.612	2065	53
2	1300	81	0.533	0.409	208	50
<i>Injected animals</i> (2 g. sodium <i>dl</i> -lactate intraperitoneally):						
3	1300	95	10.43	0.604	5704	117
4	1200	171	2.284	0.268	768	110

It will be seen that the results obtained by intraperitoneal injection parallel those resulting from intravenous injection. Also that an initial level of 5 % glycogen in the liver does not preclude a rise to 10 % following lactate administration.

In view of the rapidity with which the young rabbit forms liver-glycogen from lactic acid it is reasonable to expect a similar increase in liver-glycogen from alanine, if lactic acid be considered an intermediary product in the transformation of alanine into glucose.

Exp. 3. Three animals from the same litter were deprived of food for 24 hours before experiment. Two of the animals were injected intravenously with 2 g. of *dl*-alanine made up to 10 cc. with isotonic saline. Killed 3 hours after injection.

			Glycogen (%)		Blood-lactic acid (mg./100 cc.)
No.	Weight (g.)	Blood-sugar (mg./100 cc.)	Liver	Muscle	
<i>Control:</i>					
1	550	75	0.583	0.242	40
<i>Injected animals</i> (2 g. <i>dl</i> -alanine intravenously):					
2	525	100	0.517	0.228	37
3	550	75	0.570	0.178	44

It will be observed that there is no significant change in liver-glycogen or in muscle-glycogen. Nor is there a rise in blood-lactic acid as was observed when lactic acid was injected.

An experiment was then carried out to see what was happening to the injected *dl*-alanine. With this end in view the amino-acid and urea contents of both the blood and the urine were estimated.

Exp. 4. Five young rabbits from the same litter were taken and deprived of food for 24 hours before experiment. One animal was used as a control and into each of the others were injected 2 g. of *dl*-alanine. The animals were killed 1½, 3, 4½ and 6 hours after injection. The control animal received 10 cc. of isotonic saline intravenously. Before being injected the urinary bladders of the animals were emptied, and afterwards the animals were placed over receptacles and the urine in these and from the bladder was collected at the end of the experiment.

Weight (g.)	Killed (hours after injec- tion)	Glycogen (%)		Blood- amino-acid (mg./ 100 cc.)	Urine- amino-acid (total amount mg.)	Urine- urea (total amount mg.)	Urine- lactic acid (total amount mg.)
		Liver	Muscle				
<i>Control:</i>							
1150	3	1.67	0.262	9.39	5.48	115	0.594
<i>Injected animals (2 g. dl-alanine intravenously):</i>							
1100	1½	1.47	0.266	24.7	84.2	50	6.9
975	3	1.62	0.490	21.8	80.4	54	5.23
1300	4½	1.75	0.230	15.0	106.2	105	16.6
1150	6	1.75	0.315	13.4	98.3	192	9.76

The values obtained for blood-urea and blood-lactic acid were without significance. The remarkable constancy of the liver-glycogen values will be at once apparent. There is no significant change in muscle-glycogen. On an average less than 5 % of the injected amino-acid was excreted as such in the urine. The excretion of urea does not show the increase that might have been expected. Although there is a slight increase in lactic acid excretion, the absolute values are very small.

Having in mind the possibility that enough time had not been allowed to show glycogen deposition, the following experiment was devised.

Exp. 5. Two young rabbits from the same litter were deprived of food for 24 hours before experiment. The control animal was given three 10 cc. injections of isotonic saline at intervals of 2 hours. The other animal was given three injections containing 1 g. of *dl*-alanine in each injection, also at 2-hourly intervals. Both were killed 24 hours after the first injection. Urine was collected as in *Exp. 4*.

Weight (g.)	Glycogen (%)		Blood- amino-acid (mg./ 100 cc.)	Urine- amino-acid (total amount mg.)	Urine-urea (total amount mg.)	Urine- lactic acid (total amount mg.)
	Liver	Muscle				
<i>Control:</i>						
1050	0.282	0.136	9.3	5.25	3488	0.09
<i>dl-Alanine-injected animal (3 g.):</i>						
1025	0.303	0.240	8.1	155.2	3183	0.09

The same negative results as in *Exp. 4* were obtained again. Of 3000 mg. of *dl*-alanine injected only 155 were excreted in the urine. The lactic acid excretion in each case is an equal but negligible quantity.

Finally, to investigate the problem under conditions as nearly normal as possible the alanine was given orally.

Exp. 6. Four young rabbits from the same litter were deprived of food for 24 hours before experiment. Two were used as controls and the other two each received 4 g. of *dl*-alanine by stomach tube. The animals were killed at the end of 6 hours.

No.	Weight (g.)	Glycogen (%)		Blood- amino-acid (mg./ 100 cc.)	Urine- amino-acid (total amount mg.)	Urine- urea (total amount mg.)	Urine- lactic acid (total amount mg.)
		Liver	Muscle				
<i>Controls:</i>							
1	850	0.505	0.174	10.9	3.5	812	3.6
2	625	0.494	0.165	8.9	—	—	—
<i>dl-Alanine-administered animals (2 g. orally):</i>							
3	875	0.390	0.228	22.6	25.0	580	Nil
4	500	0.445	0.173	43.5	—	—	—

The urine secreted by Nos. 2 and 4 was insufficient for quantitative investigation. No lactic acid could be recovered from the urine of No. 3. The excretion of urea over short periods varies so much in different animals, that not much reliance can be placed on comparative figures. The foregoing experiment demonstrates the inability of the animals to lay down liver-glycogen from orally administered *DL*-alanine.

DISCUSSION.

The ease and speed with which lactic acid is transformed into liver-glycogen in the young rabbit is clearly demonstrated. But when *DL*-alanine, an amino-acid closely allied structurally to lactic acid, the immediate deamination product of which may indeed be lactic acid, is injected no increase in liver-glycogen is obtained. To explain the discrepancy between these results and the above-mentioned findings of Neuberg and Langstein it is necessary to review the work of the latter in the light of present day knowledge. In their work no mention is made of the length of starvation, the age of the animals, the time interval between the giving of the amino-acid and killing of the animal, nor what methods of control, if any, were used. Without careful attention to these very important considerations an increase in liver-glycogen may be apparent but unreal. Moreover these investigators did not estimate the muscle-glycogen. If there had been much excitation of the animals during the experiment a decrease in muscle-glycogen might have been observed; and the consequent liberation of lactic acid in the muscles could have been responsible for formation of glycogen in the liver and the substantial excretion of lactic acid which they found in the urine of their animals, amounting to about 10 % of the weight of alanine given. This is approximately the lactic acid excretion obtained in my work after injecting sodium lactate itself. When, in my experiments, alanine was given quite negligible amounts of lactic acid were obtained. It is possible that inability to demonstrate appreciable quantities of lactic acid in the urine under these conditions may indicate that lactic acid is not an intermediate product in the breakdown of alanine to glucose.

Since only up to 5 % of the injected alanine can be recovered as such in the urine, and since urea excretion figures show little evidence of any substantial deamination, it is obvious that the excess of amino-acid is taken up by the tissues.

In such conditions as diabetes and phlorrhizin poisoning the processes of gluconeogenesis are proceeding at a rate much greater than is normally called for, and the deamination of protein degradation products is so rapid that administered amino-acid is at once transformed into glucose, and thence under suitable conditions to glycogen. In the normal animal, however, the process of utilisation of amino-acid is comparatively slow and the tissues therefore take up the excess administered, and the rate of its removal from the tissues is determined by the necessities of calorie requirements.

SUMMARY.

1. The ease with which the young rabbit forms liver-glycogen from injected sodium *DL*-lactate is demonstrated.
2. Similar injections of *DL*-alanine cause no increase in liver-glycogen and this result serves to emphasise the great difference between the metabolism of amino-acids in diabetic or phlorrhizinised animals and normal animals. The most

probable interpretation of this result is that in the normal animal the tissues take up the amino-acid rapidly and release it so slowly that no appreciable deposition of glycogen can be detected.

I wish to acknowledge my thanks to Dr M. G. Goldblatt for his help and advice throughout the work.

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CLV. THE PREPARATION OF GALACTURONIC ACID FROM PLANT MATERIALS, WITH A NOTE ON SOME OF ITS DERIVATIVES.

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So far, the most satisfactory source of galacturonic acid has been a commercial "lemon pectic acid" of American origin. It was from this material that Link and Dickson [1930] obtained the acid in good yield. The commercial product is not, however, readily obtained in this country, and for this reason attempts were made to discover a suitable plant material, readily available, which could be used in place thereof.

A plant material with a high pectin content and relatively free from other polysaccharides would be ideal for the purpose. Suarez [1917] used expressed lemon pulp but was unable to obtain the acid in crystalline form. Ehrlich and Schubert [1929] used the so-called "hydrato-pectin" from beetroot, but their method was tedious and the yield poor. No other attempt seems to have been made to use plant tissues directly for the preparation of the acid.

Three materials were used in the attempt to prepare the acid:

- (1) sugar beet residues;
- (2) apple pomace;
- (3) lemon peel.

Of these, lemon peel was found by far the most satisfactory. Crude galacturonic acid was obtained from apple pomace, but it could not be separated from an unknown laevorotatory substance.

EXPERIMENTAL.

The lemon peel was cut into small pieces and boiled with 60 % alcohol for a short time, dried, and coarsely powdered. Portions of 100 g. were hydrolysed with 2000 cc. of 3 % H_2SO_4 for 20 hours, preliminary experiments having shown that a maximum yield of galacturonate was obtained under these conditions. After hydrolysis, the bulk of the sulphuric acid was removed by barium hydroxide, which was added with vigorous stirring, and finally by shaking with barium carbonate. The filtrate was evaporated to 400 cc. at a temperature below 40° , twice decolorised by shaking with norite, and from the solution the barium galacturonate was precipitated by pouring into 3 volumes of alcohol. The precipitate was well washed with warm alcohol and dried *in vacuo*. The amount obtained from 100 g. of the dry peel was 30 g., with an ash content equivalent to 26.48 % Ba. The barium salt was decomposed with sulphuric acid in the manner described by Link and Nedden [1932]. It was found that if the syrup did not crystallise within 2 days in a vacuum desiccator it was best to granulate

it by means of dry acetone; a considerable amount of brown colouring matter remained in the acetone. 10 g. of barium salt gave a yield of 3 g. of free galacturonic acid as a white crystalline powder. This could then be recrystallised from a mixture of dilute alcohol and acetone or glacial acetic acid and ether; in either case the acid was dissolved in the minimum quantity of the solvent in which it was soluble and then acetone or ether added till the solution was just turbid and allowed to crystallise. The acid was thus obtained as white crystals, M.P. 159°.

Derivatives of d-galacturonic acid.

Despite the widespread occurrence of galacturonic acid among natural products, little information had been published, at the time this work was undertaken, regarding any of its characteristic derivatives. At the suggestion of Dr H. W. Buston, the author commenced the study of certain derivatives of the acid, in order to extend the existing information in this direction. The appearance of a paper [1933] by Niemann *et al.*, in which they describe a number of hexuronic acid derivatives, rendered a continuance of the investigation unnecessary. It may be worth while, however, to record details of the preparation of the two derivatives which, in the opinion of the author, are the most useful for the purpose of characterising *d*-galacturonic acid, and which had been prepared and studied before the publication of the paper of Niemann *et al.*

(a) *Cinchonine d-galacturonate*. This is the most useful salt for identifying galacturonic acid, when it occurs as a constituent of plant products. It has a special advantage over some other derivatives in that it may be prepared directly from the barium salt of galacturonic acid, thereby rendering it unnecessary to obtain the acid in the free solid condition.

Of the methods of preparation tried, the following gave the most satisfactory results. 1 g. of dry barium galacturonate is suspended in 100 cc. absolute alcohol and 1.3 g. cinchonine sulphate (slightly less than the calculated quantity) dissolved in alcohol are added. The mixture is then mechanically shaken for 4-5 hours, warmed at 40-50° for an hour and filtered when the solution is still warm, to remove the precipitate of barium sulphate and any unchanged barium galacturonate. The precipitate is then washed once or twice with warm alcohol. The mixed filtrate is evaporated almost to dryness *in vacuo* at 50°, when cinchonine galacturonate readily crystallises out. It is triturated two or three times with small quantities of absolute alcohol to remove any unchanged cinchonine sulphate. Recrystallisation is best effected by dissolving the salt in the minimum quantity of hot alcohol, filtering and adding excess of ether or acetone until a distinct turbidity appears. The solution is then made clear again by gentle warming and allowed to crystallise in a refrigerator. The salt crystallises out in rosettes of tiny white needles in a few hours.

Properties. The salt melts at 173° (with decomposition)¹. (Found: C, 61.4; H, 6.7; N, 5.7 %. $C_6H_{10}O_7$, $C_{19}H_{22}N_2O$ requires C, 61.4; H, 6.6; N, 5.7 %.)

It is very sparingly soluble in cold, but dissolves readily in warm alcohol. It is quite insoluble in other organic solvents such as benzene, chloroform, and ether.

(b) *Phenylhydrazone of phenylhydrazine d-galacturonate*. Ehrlich was unable to prepare any phenylhydrazine derivatives from *d*-galacturonic acid, probably for reasons to be stated later. Niemann *et al.* [1933] report the preparation of a

¹ Ehrlich records two different melting-points, 156 and 178°. Also it is to be noted that his analysis shows a molecule of water of crystallisation in the salt. The variation of the physical properties of the cinchonine salts of hexuronic acids is commented upon by Niemann *et al.* [1933].

number of derivatives of phenylhydrazine, including the phenylhydrazone of phenylhydrazine *d*-galacturonate. This, in the experience of the author, is an extremely characteristic derivative; its preparation, however, necessitates the isolation of the acid in a free solid state and hence it is a less convenient substance to prepare than the cinchonine salt.

Niemann *et al.* report a 60 % yield of the derivative. By the following method the yield exceeds 90 %. A 5 % aqueous solution of the acid is treated with excess of phenylhydrazine acetate (1 g. acid, 1.2 g. phenylhydrazine, 1 g. 50 % acetic acid) and warmed at 40–50° for not more than 5 minutes. On cooling, the phenylhydrazone of phenylhydrazine galacturonate crystallises in rosettes of very flat elongated prisms of a pale yellow colour. More prolonged heating decomposes the derivative, with the production of brown oily substances; probably this explains the failure of Ehrlich to obtain any phenylhydrazine compounds from the acid. It is best recrystallised by suspending in boiling chloroform and adding hot methyl alcohol drop by drop until the solid almost dissolves. The solution is filtered and allowed to crystallise. The m.p. 131° (uncorr.) with decomposition was in agreement with that of Niemann *et al.* (Found: C, 55.2; H, 6.1; N, 14.2 % (Schoeller). $C_{18}H_{22}O_5N_4 \cdot H_2O$ requires C, 55.1; H, 6.2; N, 14.3 %.)

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CLVI. THE ABSORPTION SPECTRA OF THE MIXED FATTY ACIDS FROM COD-LIVER OIL.

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(Received July 3rd, 1933.)

A SURPRISING difference between the absorption spectrum of cod-liver oil itself and that of the mixed fatty acids derived from it has been recorded by Heilbron and Morton and their co-workers. Cod-liver oil itself [Morton and Heilbron, 1928] was found to have relatively low absorption ($E_{1\text{ cm.}}^{1\%} = 1.2$ for a good oil) in the ultra-violet, characterised by the broad unbroken band of vitamin A at 3280 Å. The mixed acids and unsaponifiable matter, on the other hand [Gillam *et al.*, 1931], as obtained by acidification and ether extraction of the hydrolysate, had so intense an absorption ($E_{1\text{ cm.}}^{1\%}$ at 2700 Å. = 250, at 2300 Å. = 190) as to obscure the presence of the admixed vitamin A. The absorption moreover was characterised by fine structure showing not only a band at 2300 Å., which these workers state is shown by a wide range of unsaturated substances, but also no less than 9 narrow absorption bands at 3920, 3750, 3500, 3300, 3160, 3020, 2810, 2700 and 2590 Å. Since the re-esterification of the acids with methyl alcohol or glycerol did not lead to a reversion to the weak absorption shown by the original oil, the change was obviously irreversible, and it was suggested, as the simplest explanation in consonance with all the facts then observed, that under the conditions of hydrolysis a substance (or substances) accompanying vitamin A gives rise to acid decomposition products which display intense selective absorption.

In connection with joint work with the National Institute for Research in Dairying we recently had occasion to carry out a spectroscopic examination on a sample of cod-liver oil which was to be used for feeding to cows. As a result of this work we have encountered a phenomenon which appears to have been overlooked by the Liverpool workers, and which may help to some extent in explaining their observations. According to our experiments the duration of the saponification process exerts such a profound influence upon the absorption spectrum of the acids as to make data obtained under uncontrolled conditions of saponification almost meaningless.

EXPERIMENTAL.

Throughout the present work a Hilger Type E 3 Quartz Spectrograph was used. Specially purified cyclohexane (B.D.H.) was used as solvent.

The ultra-violet absorption of the mixed fatty acids obtained from cod-liver oil by brief saponification. The oil first examined was a crude specimen as used for veterinary purposes. 5 g. of this oil were saponified by heating for about 1 minute with 2.2 cc. of saturated aqueous KOH and 10 cc. of ethyl alcohol. (These

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proportions were used in all the saponifications described below.) The soaps were freed from unsaponifiable matter by extracting 3 times with ether and the free acids obtained by acidification with slight excess of 10 % H_2SO_4 followed by ether extraction. On spectroscopic examination of the mixed acids an absorption maximum was found at 2300 Å., but the value of $E_{1\text{ cm.}}^{1\%}$ was only 7.7.

Since this value was only one twenty-fifth as great as that found by Gillam and his colleagues the possibility occurred to us that the oil might be an abnormal specimen. We therefore repeated the experiment using Engelberg's Norwegian cod-liver oil, a well-known medicinal brand. $E_{1\text{ cm.}}^{1\%}$ at 2300 Å. for the mixed acids was 6.25, a slightly lower value than that for the crude oil.

Absorption of the mixed fatty acids obtained after prolonged saponification. The Engelberg oil, which we used in all subsequent experiments, was next saponified by boiling with the alcoholic KOH on a water-bath for 4½ hours, the unsaponifiable matter then being removed by two extractions with ether and the free acids obtained in the usual way. $E_{1\text{ cm.}}^{1\%}$ 2300 Å. was now found to be 62.5, having thus increased tenfold as the result of the prolongation of the saponification.

The effect of potassium hydroxide on the absorption of the mixed fatty acids. Since Gillam and his colleagues suggest that the high absorption observed by them in the mixed acids may be due to acid decomposition products, presumably derived from the non-saponifiable residue, our next step was to examine the effect of boiling with alcoholic KOH on mixed acids freed from non-saponifiable material. In a preliminary experiment the acids from Engelberg cod-liver oil, $E_{1\text{ cm.}}^{1\%}$ 2300 Å. = 6.25, obtained in one of the experiments described above, were boiled with alcoholic KOH for two periods totalling 4½ hours over a total period of 19 hours. As the result of this treatment $E_{1\text{ cm.}}^{1\%}$ 2300 Å. increased to 110.

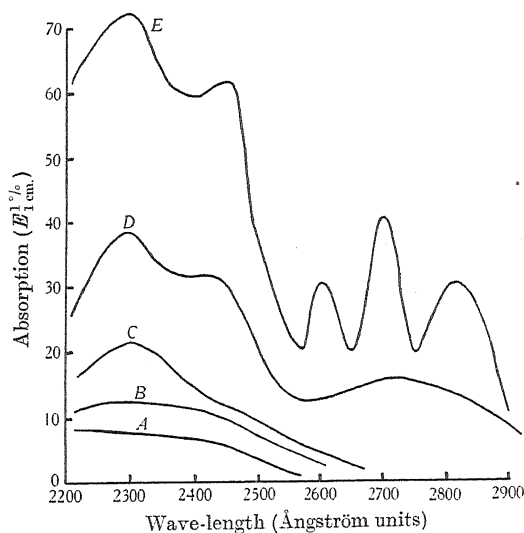


Fig. 1. Absorption spectra of mixed fatty acids from cod-liver oil.

- A. Mixed fatty acids obtained after saponification lasting 1 minute.
 B. Same acids after treatment with boiling alcoholic KOH for 15 minutes.
 C. Same acids as A, after treatment with boiling alcoholic KOH for 1 hour.
 D. " " " " " " " " " " 4½ hours.
 E. " " " " " " " " " " 12 hours.

The experiment was repeated using a further portion of the oil. In this case the unsaponifiable matter obtained after 1 minute's saponification was separated by 3 ether extractions and preserved for examination. The free acids were then redissolved in alcoholic KOH and heated on a water-bath, samples being taken after periods up to 12 hours. The free acids were obtained by the usual procedure and examined spectroscopically with the following results:

Time of heating with alcoholic KOH	1 min.	15 mins.	1 hr.	4.5 hrs.	12 hrs.
$E_{1\text{ cm.}}^{1\%}$ at 2300 Å. for the free acids	7.6	12	22	39	72

The absorption was again greatly increased as the result of heating with the alkali. Moreover in the case of the acids treated for 4.5 and 12 hours discontinuity in the bands was evident, suggesting the presence of fine structure formation. The shapes of the absorption curves obtained are roughly indicated in Fig. 1.

The effect of potassium hydroxide on the absorption of the non-saponifiable residue of cod-liver oil. The unsaponifiable matter obtained from the 10 g. of oil used in the above experiment amounted to about 130 mg. It was divided into two equal portions. One portion was examined spectroscopically and also by the SbCl_3 reaction, without further treatment, the other was boiled for 4 hours with alcoholic KOH and then re-extracted before examination. The following results were obtained:

N.S.R. equivalent to 5 g. C.L.O.

	Wt. of residue mg.	B.U. per mg. (SbCl_3 test)	$E_{1\text{ cm.}}^{1\%}$ at		
			3280 Å.	2600 Å.	2300 Å.
1 min. boiling with alcoholic KOH	70	250	62	17	38
4 hrs. boiling	66	250	69	34	55

Definite increases of absorption at 2300 Å. and 2600 Å. were therefore observed, but the amount either after 1 minute or 4 hours would represent an altogether negligible contribution in attempting to account for the high absorption observed by Gillam and his colleagues in the combined acids and non-saponifiable residue. On the other hand the vitamin A content according to the SbCl_3 test, remained unchanged. Since, moreover, there was no loss in weight outside the range of experimental error as the result of boiling, it appeared probable that the non-saponifiable residue as a whole showed little tendency to form acid decomposition products under the particular treatment adopted, and no attempt was therefore made to isolate acidic products from the aqueous layer.

Absorption spectra of stearic, oleic and linoleic acids. To obtain some clue to the type of acid taking part in the change leading to increased absorption we have examined the absorption spectra of crude specimens of stearic, oleic and linoleic acids both before and after boiling with alcoholic KOH. Results were as follows:

	$E_{1\text{ cm.}}^{1\%}$ 2300 Å.	$E_{1\text{ cm.}}^{1\%}$ 2300 Å. after 4 hrs. heating with alcoholic KOH
	Initial	
Stearic acid	0.9	1
Oleic acid	16.5	18
Linoleic acid	9.6	38

It will be seen that the absorption of stearic acid and oleic acid was unaffected by the treatment with KOH. The absorption of linoleic acid on the other hand, although initially lower than that of oleic acid, was increased about 4 times after heating with alcoholic KOH.

DISCUSSION.

As far as can be judged from experiments on a single typical specimen of cod-liver oil the above work indicates that the mixed acids obtained as the result of a brief saponification possess relatively low absorptive powers, but that on more prolonged heating with alcoholic KOH they undergo some change which results in much higher absorption.

Whether this phenomenon can be regarded as a complete explanation of the observations of Gillam and his colleagues cannot be decided with certainty, since these workers have given no details of their technique in saponification. As evidence in favour of the existence of two separate phenomena, or overlapping phenomena, it might be argued that in our experiments heating for 12 hours with alcoholic KOH produced only about half the intensity of absorption observed by the Liverpool workers, while fine structure was slightly less fully defined. Such differences might lead us to suspect that cod-liver oils differ fundamentally in their behaviour after saponification from specimen to specimen. In our opinion, however, the cause of the divergence is much more likely to be traceable to differences in the technique adopted for the saponification of the oils and the separation of the acids.

In regard to the chemical nature of the change taking place on heating with alcoholic KOH our experiments on stearic, oleic and linoleic acids appear to indicate that the acids involved must possess two or more double bonds, since only in the case of linoleic acid was increased absorption observed as the result of this treatment. Moreover preliminary iodine value determinations, carried out both on linoleic acid and the mixed acids of cod-liver oil, failed to reveal any significant change in the degree of unsaturation as a result of boiling. It seems possible, therefore, that the increased absorption may be related to an isomeric change among the unsaturated linkages of the more highly unsaturated acids.

From a biological standpoint our results may be of interest in opening up the possibility that highly unsaturated acids may exist *in vivo* in two forms, non-absorptive and absorptive. In an extension of joint work on butter [Booth *et al.*, 1933] we have already obtained evidence which suggests strongly that this may be the case. We hope later to examine non-absorptive and absorptive acids with a view to detecting changes in their chemical behaviour, particularly in their ability to form crystalline bromine derivatives and in their oxygen uptake.

SUMMARY.

The mixed fatty acids prepared from a typical cod-liver oil by a brief saponification, removal of the non-saponifiable matter by ether, acidification and ether extraction showed only relatively low absorption in the ultra-violet region without fine structure ($E_{1\text{ cm}}^{1\%}$ 2300 Å. = 6.5). If however the time taken for the saponification were prolonged, or if the separated acids were boiled with a further supply of alcoholic KOH, the absorption became much more intense ($E_{1\text{ cm}}^{1\%}$ 2300 Å. after 12 hours = 72) and definite signs of fine structure became evident.

Our thanks are due to Dr L. J. Harris for his valuable criticism.

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CLVII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXII. CYNODONTIN (1:4:5:8-TETRAHYDROXY-2-METHYLANTHRAQUINONE), A METABOLIC PRODUCT OF *HELMINTHOSPORIUM CYNODONTIS* MARIGNONI AND *HELMINTHOSPORIUM EUCHLAENAE* ZIMMERMANN.

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(Received June 16th, 1933.)

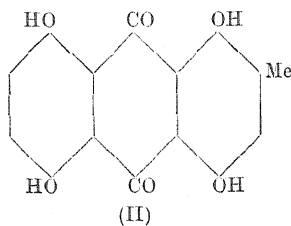
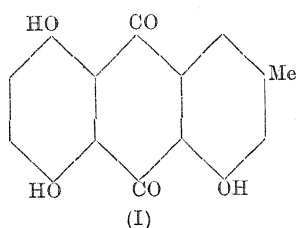
HELMINTHOSPORIUM CYNODONTIS Marignoni was first described by Marignoni [1909] as occurring on dry leaves of *Cynodon Dactylon* Persoon, found near Schio in Northern Italy. The species was apparently not reported again until Drechsler [1923], in a monograph on the graminicolous species of *Helminthosporium*, refers to it in considerable detail. *Cynodon Dactylon*, the host plant, is the so-called "creeping dog's-tooth grass" which is found on sandy shores in the south-west of England, the Bermuda grass of the southern United States, and the "durba" grass of India. It forms an important forage grass in many parts of America and India. Drechsler reports that "the parasite *Helminthosporium cynodontis* is exceedingly common throughout the south-eastern section of the United States where the host is everywhere present in the fields and on the roadsides as a noxious weed."

Helminthosporium euchlaenae Zimmermann was described by Zimmermann [1904, 1, 2] as occurring on the leaves of *Euchlaena mexicana* Schrad (= *E. luxurians* Dur. and Ashers. = *Reana luxurians* Dur.). This host plant is an annual grass with a habit like maize, growing to a height of 5-10 ft. or more, and is occasionally cultivated in the States on the Gulf of Mexico as a forage cereal, where it is known by the name of "Teosinte."

The strain of *Helminthosporium cynodontis* used in the work about to be described was purchased in May 1932 from the Centraalbureau voor Schimmelcultures, Baarn, Holland, and was received by them in January 1929 from Dr Charles Drechsler of the Bureau of Plant Industry, United States Department of Agriculture, Washington. It is very probably the strain described by Drechsler [1923] in his monograph, and isolated by him from diseased Bermuda grass.

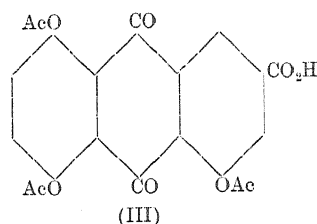
The strain of *Helminthosporium euchlaenae* used by us was purchased in May 1932 from Baarn, where it was isolated in May 1929 by Dr van Beyma thoe Kingma as a contamination of a culture of *Pythium* received by the Centraalbureau voor Schimmelcultures from Sideris, Hawaii, in April 1929.

When these two fungi were grown on a purely synthetic medium containing glucose as the sole source of carbon, good growth was obtained in each case, the chief obvious characteristic of which is the chocolate-brown colour of the reverse of the mycelium. The dried mycelium on extraction with chloroform gave in the case of *H. cynodontis* a considerable quantity, and in the case of *H. euchlaenae* a smaller quantity, of a substance crystallising in bronze-coloured plates and having the appearance of bronze powder. This substance, which we propose to term *cynodontin*, has the empirical formula $C_{15}H_{10}O_6$ and has not previously been described. From its origin and general properties it was considered to be a near relative of helminthosporin (I) which Charles, Raistrick, Robinson and Todd [1933] described as a metabolic product of the plant pathogen *Helminthosporium gramineum* Rabenhorst, and the constitution of which has been settled by synthesis [Raistrick, Robinson and Todd, 1933].



Actually it was found possible to convert helminthosporin ($C_{15}H_{10}O_5$, 4:5:8-trihydroxy-2-methylantraquinone) into cynodontin ($C_{15}H_{10}O_6$) by means of manganese dioxide and sulphuric acid. The substance is not a powerful mordant dyestuff and hence the entering hydroxyl should be in the 1-position. Cynodontin is therefore, in all probability, 1:4:5:8-tetrahydroxy-2-methylantraquinone (II).

The extracted mycelium from *H. cynodontis* afforded in addition to cynodontin, but in small relative amount, a colouring matter having the colour reactions of helminthosporin; this was not completely purified but its presence was demonstrated by conversion into triacetylhelminthosporic acid (III). The product from *H. euchlaenae* consisted of practically pure cynodontin.



EXPERIMENTAL.

Preparation of crude pigment. 35 litres of Czapek-Dox medium of the following composition were made up: glucose, 50.0 g.; $NaNO_3$, 2.0 g.; KH_2PO_4 , 1.0 g.; KCl , 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $FeSO_4 \cdot 7H_2O$, 0.01 g.; distilled water, 1000 cc. 350 cc. quantities of this medium were distributed in each of 100 1-litre conical flasks and sterilised. A measured quantity, previously determined, of sterile *N* sodium hydroxide solution was then added to each flask to bring the p_H of the medium to 8.0. Each flask was sown with a suspension, in sterile Czapek-Dox medium, of *Helminthosporium cynodontis* Marignoni (Catalogue No. Ag. 120).

The cultures used for inoculation had been grown for about 3 weeks on Czapek-Dox agar at 30°. After inoculation the flasks were incubated at 30° for 7 weeks. At the end of this time the mycelium, which was greyish on the surface with a chocolate-brown reverse, was filtered off, washed carefully with cold water, pressed and dried in a vacuum-oven. The dried mycelium in two experiments weighed 230 g. in each case. It was ground to a fine powder in a coffee-mill and exhaustively extracted with chloroform in a large Soxhlet apparatus, until the chloroform extract which, in the early stages was of a beautiful eosin-red colour, had become almost colourless. On cooling there separated from the chloroform solution (about 1½ litres) in a typical experiment 7.5 g. of bronze-coloured plates having a melting-point of 258–260°. The chloroform mother-liquors were evaporated to about 250 cc. and from this there separated a second fraction weighing 1.25 g. and melting at 250–255°. The filtrate from this fraction was evaporated to dryness, dried *in vacuo*, and extracted thoroughly with light petroleum (B.P. 40–50°) giving a third fraction which weighed 0.55 g. and melted at 208–218°. The light petroleum solution on evaporation yielded 47.6 g. of residue consisting mainly of fat together with very small amounts of pigmented material. The total yields of crude pigments in the two experiments referred to above were 9.30 and 9.74 g., while the total yields of crude fat were 47.6 and 49.6 g.

The aqueous metabolism solution separated by filtration from the mycelium and still containing about 0.4 % of glucose was evaporated *in vacuo* to a total volume of about 1 litre, giving a very dark-coloured turbid liquid. This was filtered and acidified slightly with concentrated hydrochloric acid, and four volumes of absolute alcohol were added to it with constant shaking. There thus separated a considerable quantity of gummy material, examination of which has shown it to be a carbohydrate. Further work on this material is in progress.

In experiments using *Helminthosporium euchlaenae* Zimmermann (Catalogue No. Ag. 116), the cultural conditions followed, *i.e.* medium used, incubation temperature and period of incubation, were the same as those previously described for *H. cynodontis*. The method of isolating the pigment was also the same, but the yields obtained were somewhat different. The dried mycelium in two experiments weighed 247 and 264 g. respectively, and total yields of crude pigment of 2.36 and 5.06 g. were obtained in the form of bronze-coloured plates melting between 256 and 259°. The total yields of crude fat in the two experiments were 33.9 and 37.4 g. A considerable amount of gummy material which has proved to be a carbohydrate was also isolated from the evaporated aqueous metabolism solution by a method similar to that employed with *H. cynodontis*.

Products from Helminthosporium cynodontis.

Cynodontin. Fraction I of the crude coloured material (M.P. 258–260°) from *H. cynodontis* furnished, after one recrystallisation from pyridine, pure cynodontin, M.P. 260°, unchanged by further recrystallisation. It crystallises in brownish leaflets with a magnificent bronze lustre. Like helminthosporin it is sparingly soluble in most organic solvents to give solutions which are slightly bluer in tone than those of the former substance. It is insoluble in cold dilute sodium carbonate solution but dissolves in aqueous sodium hydroxide to a deep blue-violet solution. Solutions in concentrated sulphuric acid are blue and have a fine red fluorescence. Cynodontin has practically no dyeing properties. (Found: C, 63.1; H, 3.4. $C_{15}H_{10}O_6$ requires C, 62.9; H, 3.5 %.) Fraction II of the crude material had M.P. 250–255° and consisted essentially of cynodontin.

Cynodontin was found to be identical in every respect with a specimen of 1:4:5:8-tetrahydroxy-2-methylantraquinone prepared by the oxidation of helminthosporin (see below).

Tetra-acetylcynodontin. Cynodontin (0.5 g.) was acetylated by heating to boiling for a few minutes with acetic anhydride containing a trace of sulphuric acid. After pouring into water the precipitated acetate was recrystallised several times from glacial acetic acid, when it was obtained as a mass of bright yellow needles, M.P. 224–225°. (Found: C, 60.9; H, 4.1. $C_{23}H_{18}O_{10}$ requires C, 60.8; H, 4.0 %.)

1:4:5:8-Tetrahydroxy-2-methylantraquinone from helminthosporin.

Helminthosporin (2 g.) was dissolved in concentrated sulphuric acid (15 cc.), and finely powdered manganese dioxide (1.5 g.) added gradually with constant stirring, the temperature being maintained between 50 and 60°. When all had been added the mixture was kept at 50–60° for a further 15 minutes and then poured into water; the brownish-violet precipitate produced in this way became reddish-brown (hydrolysis) on boiling for a short time with water. The product after recrystallisation from pyridine still contained unchanged starting material. Accordingly it was acetylated with acetic anhydride containing a trace of sulphuric acid in the usual manner and the acetate purified. After four recrystallisations from glacial acetic acid the melting-point of the acetate became constant at 224–225° (mixed M.P. with helminthosporin acetate, of M.P. 223–224°, *ca.* 194°). The purified acetate (0.6 g.) was warmed on the water-bath with 15 % sodium hydroxide solution (30 cc.) during 2 hours when it dissolved slowly to a deep blue-violet solution. After acidifying with hydrochloric acid and boiling for a few minutes to coagulate the precipitate, the latter was collected and recrystallised from pyridine. It separated as brownish leaflets with a fine bronze lustre, M.P. 260°. (Found: C, 63.1; H, 3.5. $C_{15}H_{10}O_6$ requires C, 62.9; H, 3.5 %.) This material showed all the properties of cynodontin and a mixed M.P. with the latter substance showed no depression.

That this product is indeed 1:4:5:8-tetrahydroxy-2-methylantraquinone is clear from the evidence already mentioned and also for the following reasons. The new hydroxyl group cannot occupy a β -position in the anthraquinone nucleus, as the oxidation product is insoluble in cold sodium carbonate solution and is not a derivative of alizarin, being an exceedingly feeble dyestuff.

In its colour reactions the oxidation product closely resembles 1:4:5:8-tetrahydroxyanthraquinone as described by Fischer and Ziegler [1912].

2-Methyl-1:4:5:8-tetra-acetoxyanthraquinone. The purified acetate obtained from the crude oxidation product of helminthosporin had M.P. 224–225° and crystallised in bright yellow needles. The same product was obtained by acetylating pure 2-methyl-1:4:5:8-tetrahydroxyanthraquinone with acetic anhydride and a trace of sulphuric acid. (Found: C, 60.7; H, 4.2. $C_{23}H_{18}O_{10}$ requires C, 60.8; H, 4.0 %.)

This acetate was identical in appearance with cynodontin acetate and the mixed M.P. with the latter substance showed no depression.

Examination of fraction III of crude material from H. cynodontis.

Fraction III (0.55 g.), which was obtained by evaporation of the chloroform mother-liquors after removal of fraction II, had an indefinite M.P. 208–218°. It appeared slightly lighter in colour than the earlier fractions and the colours of its solutions in concentrated sulphuric acid, sodium hydroxide and in organic

solvents were intermediate between those shown by cynodontin and helminthosporin. Indeed, it was possible to simulate the colours produced by fraction III by adding to solutions of cynodontin small amounts of helminthosporin.

Several attempts were made to isolate pure helminthosporin from fraction III by fractionation with various organic solvents, but with little success. Fractional crystallisation from acetic acid of the material obtained on acetylation was also tried and would doubtless have succeeded had larger quantities of material been available; thus the more soluble fractions yielded after repeated treatment a product whose reactions were more like those of helminthosporin acetate than cynodontin acetate. Its solution in sulphuric acid was reddish-violet in colour (still bluer than pure helminthosporin solutions) with the pronounced fluorescence characteristic of helminthosporin; the m.p. of this material was 210–215° but this was not depressed on mixing with a small quantity of helminthosporin acetate (m.p. 223–224°).

Confirmation of the view that fraction III contained helminthosporin was obtained in the following way. The original mixture (0.2 g.) was acetylated in the usual manner, the crude acetate dissolved in a mixture of acetic anhydride (4 cc.) and acetic acid (4 cc.) and oxidised at 90–100° by means of chromic anhydride (0.4 g.) in acetic acid (4 cc.). When the yellow precipitate obtained on diluting the reaction mixture with water was agitated with cold dilute sodium carbonate solution, a portion dissolved to a reddish-violet solution leaving behind unchanged cynodontin acetate (it had previously been ascertained that cynodontin acetate was practically unaffected by chromic anhydride under the conditions here employed). After filtering and acidifying the clear solution the reddish precipitate (30 mg.) was collected, re-acetylated by means of acetic anhydride containing a trace of sulphuric acid, and recrystallised several times from acetic acid. In this way a small quantity of material was obtained which showed all the properties of triacetylhelminthosporic acid (III). The m.p. of this product was low (230–232°) and owing to the very small quantity available further purification was impossible. The mixed m.p. with triacetylhelminthosporic acid (m.p. 235°) was 234°, leaving no room for doubt as to its identity.

From the evidence available it seems reasonable to conclude that helminthosporin is a constituent of the coloured products of *H. cynodontis*, although the amount present is very small indeed.

Products from Helminthosporium euchlaenae.

The coloured metabolic product proved to be almost homogeneous and yielded on recrystallisation pure cynodontin, m.p. 260°. (Found: C, 62.8; H, 3.8. $C_{15}H_{10}O_6$ requires C, 62.9; H, 3.5 %.) Its identity was confirmed by direct comparison with an authentic specimen of cynodontin; the appearance, colour reactions, m.p. and mixed m.p. of the specimens were identical as also were those of their acetates (m.p. and mixed m.p. 224–225°).

SUMMARY.

The mycelium of *Helminthosporium cynodontis* Marignoni and of *Helminthosporium euchlaenae* Zimmermann, when grown on a synthetic culture medium containing glucose as the sole source of carbon, has been shown to contain a hitherto undescribed, crystalline colouring matter, which has been called cynodontin. Cynodontin has been shown to be 1:4:5:8-tetrahydroxy-2-methyl-anthraquinone and is thus 1-hydroxyhelminthosporin. Helminthosporin, which

has recently been shown to be present in considerable amounts in the mycelium of the plant pathogen *Helminthosporium gramineum* Rabenhorst, is also present in very small amounts in the mycelium of *Helminthosporium cynodontis*, but is absent from *Helminthosporium euchlaenae*.

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CLVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXIII. THE MYCELIAL CONSTITUENTS OF *PENICILLIUM BREVI-COMPACTUM* DIERCKX AND RELATED SPECIES.

PART I. ERGOSTERYL PALMITATE.

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(Received July 4th, 1933.)

IN the course of the studies reported in Part XXIV of this series [Clutterbuck *et al.*, 1932] considerable amounts of mycelia from the growth on synthetic media of 15 species and strains in the *P. brevi-compactum* series were accumulated, and in view of the close biochemical relationship of 14 of these organisms as regards their water-soluble metabolic products (see Part XXIV) it became a matter of some interest to investigate their mycelial constituents in order to discover whether this biochemical relationship is of even wider scope. The mycelium of the type species *P. brevi-compactum* Dierckx (Cat. No. P 75) was first investigated, the method being to extract the dried and powdered mycelium with a succession of solvents, *viz.* light petroleum followed by ether, then by acetone and finally by methyl alcohol. This paper is concerned only with the mycelial fat, *i.e.* the material extracted from the mycelium by hot light petroleum, B.P. 50–60°.

It was noticed that a considerable part of the mycelial fat from this species was not soluble in cold absolute alcohol, the insoluble constituent giving all the colour reactions of ergosterol. This insoluble substance, which was much more sparingly soluble in most organic solvents than ergosterol itself, had M.P. 106–108°; $[\alpha]_{5461}^{20^\circ} - 67^\circ$, $[\alpha]_{5790}^{20^\circ} - 52^\circ$ when pure, and could not be made to yield a benzoyl or an acetyl derivative. Its carbon and hydrogen content and molecular weight corresponded to an empirical formula of the order of $C_{44}H_{74}O_2$ and the substance was evidently an ergosteryl ester. When hydrolysed by alcoholic potash it yielded ergosterol and a fatty acid identified as palmitic acid, and was therefore clearly ergosteryl palmitate, $C_{44}H_{74}O_2$, a substance which had previously been prepared synthetically by Windaus and Rygh [1928] by heating ergosterol and palmitic acid *in vacuo* at 190°. The physical constants quoted by these authors, *viz.* M.P. 107–108°, $[\alpha]_{5890}^{18^\circ} - 51^\circ$ are in good agreement with those of the mould product quoted above.

The same substance, or at any rate a substance melting at about 100° whose melting-point was not depressed on admixture with the product from *P. brevi-compactum* (P 75), was obtained from the mycelium of each of the 14 remaining species and strains of the *P. brevi-compactum* series investigated, with the solitary exception of *P. stoloniferum* Thom (Ad 89). The yield of recrystallised ergosteryl palmitate was usually of the order of 0.02 % of the dry weight of

mycelium but in one solitary instance, viz. *P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski, the yield of purified ester was as high as 0.5 % and corresponded to 0.06 % of the glucose metabolised in the experiment during which the mycelium was formed. This ester also yielded ergosterol and palmitic acid on hydrolysis.

It is worthy of note that this appears to be the first recorded instance of the isolation of an ergosteryl ester in a pure state from a natural source, although the results of Smedley-MacLean and Thomas [1920], recorded some 13 years ago, indicated clearly that a large proportion of the ergosterol in yeast-fat was present in the form of esters. King *et al.* [1929] showed that the cholesterol esters extracted from a mummified brain 14 centuries old were undoubtedly admixed with ergosterol esters. Pruess *et al.* [1931] estimated the total sterol content of the mycelia of a number of moulds and showed that while most of the alcohol-soluble sterol was present in the free state in the extract, a definite, and in some cases a considerable, proportion of the total sterol in the mycelium was in a form which was not extracted by alcohol, but which yielded an alcohol-soluble sterol on hydrolysis by alcoholic potash. These authors concluded that this latter sterol is probably present in a conjugated form. Birkinshaw *et al.* [1931] found that a considerable fraction of the crude sterol obtained from the mycelium of *P. puberulum* Bainier grown on a synthetic medium containing glucose as the sole source of carbon could not be precipitated by digitonin, although the reaction with $\text{CCl}_3\text{CO}_2\text{H}$ was strongly positive, and concluded that this fraction contained ergosterol largely as ester.

It appeared unlikely therefore that ergosteryl palmitate would prove to be a mycelial constituent specific for the *P. brevi-compactum* series, and it has, in fact, been isolated in relatively good yield (0.4 %) from the mycelium of *P. italicum* Wehmer grown on liquid Czapek-Dox medium at 30°. The yield of this ester from the mycelium of *P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski (Cat. No. P 69) would, however, appear to be exceptionally high (the greater part of the sterol in the mycelium being present as palmitate) and it may be significant that although this organism is morphologically definitely a member of the *P. brevi-compactum* series [see Clutterbuck *et al.*, 1932, p. 1443], yet biochemically it has no affinities with the other members of this group, since, unlike them, it yields no trace of mycophenolic acid or of any of the other phenolic acids, $\text{C}_{10}\text{H}_{10}\text{O}_5$, $\text{C}_{10}\text{H}_{10}\text{O}_6$, $\text{C}_{10}\text{H}_{10}\text{O}_7$ and 3:5-dihydroxyphthalic acid when grown on Raulin-Thom medium [see Clutterbuck *et al.*, 1932, p. 1446].

EXPERIMENTAL.

The sources of the organisms employed are given in Part XXIV [Clutterbuck *et al.*, 1932, p. 1443]. Each was grown on Raulin-Thom medium containing both glucose and tartaric acid as sources of carbon, and ammonium tartrate, phosphate and sulphate as sources of nitrogen. The temperature of incubation was 24° and the period of incubation was 20–30 days, the experiment being concluded when all the sugar in the medium had been utilised. The mycelium was filtered off, washed, pressed and dried in a vacuum-oven. The mycelium was ground to a fine powder in a coffee-mill and extracted.

Isolation of ergosteryl palmitate from the dried mycelium. The usual procedure was to extract 50 g. of powdered mycelium with light petroleum (B.P. 50–60°) for 2 working days (15 hours) in an all-glass Soxhlet apparatus. The extract, filtered from a little mycophenolic acid if necessary, was evaporated to small volume and the remaining solvent removed *in vacuo*. The residual yellow fat

was usually of a fairly hard consistency, but the mycelium of *P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski (Cat. No. P 69) yielded a very dark coloured and much more fluid fat. Cold absolute alcohol (20 cc. per g. of crude fat) was added and the flask shaken until no fat particles remained adhering to the walls of the flask. The insoluble residue was filtered off, washed twice with alcohol, dried in air and recrystallised from the minimum volume of boiling acetone, about 40 cc. being required for each 0.1 g. of residue. The results for the various mould mycelia are summarised in Table I, the yields in each experiment

Table I.

Catalogue No.	Species [see Clutterbuck et al., 1932, p. 1443]	Weight of dried mycelium g.	Ergosteryl palmitate (once crystallised from acetone)		
			Yield in g.	Yield % of mycelium	M.P. and remarks
P 75	<i>P. brevi-compactum</i> Dierckx	276	0.25	0.1	102–104° (a), (b)
M 3 (1)	Unnamed. Isolated in 1931 from mouldy Italian maize	386	Trace	—	100–102° (a), (b)
M 3 (3)	Unnamed. Isolated in 1931 from mouldy Italian maize	285	Trace	—	100–102° (a), (b), (c)
M 3 (4)	Unnamed. Isolated in 1931 from mouldy Italian maize	388	Trace	—	(a)
P 151	<i>P. scabrum</i> Biourge	243 (fat content 0.6 %)	Trace	—	92–96° (a)
A 11	<i>P. brevi-compactum</i> Dierckx. Isolated from cotton	419	Trace	—	94–100° (a), (c)
S 30	Unnamed. Isolated from soil	293	0.06	0.02	100–103° (a), (b), (c)
P 84	<i>P. Hagemi</i> Zaleski	450	0.09	0.02	97–102° (a), (b), (c)
P 99	<i>P. Szaferi</i> Zaleski	446	0.05	0.01	102–104° (a), (b), (c)
D 8	Unnamed. Plate contaminant	432	0.13	0.03	98–103° (a), (b), (c)
P 90	<i>P. palris-mei</i> Zaleski	334	0.17	0.05	102–104° (a), (b), (c)
P 37	<i>P. griseo-brunneum</i> Dierckx	524	0.23	0.04	96–100° (a), (b), (c)
Ad 89	<i>P. stoloniferum</i> Thom	547	None. Fat completely soluble in alcohol		
Ad 87	<i>P. Biourgeanum</i> Zaleski	363	0.06	0.02	98–101° (a), (b), (c)
P 69	<i>P. aurantio-griseum</i> Dierckx, var. <i>Poznaniensis</i> Zaleski	229 (d) (fat content, 8 %)	1.14	0.50	102–105° (a), (b), (c)
Ad 85	<i>P. italicum</i> Wehmer. Isolated from a mouldy orange in 1926	195 (e) (fat content, 5 %)	0.80	0.41	102–104° (a), (b), (c)

(a) Gives positive reaction with Callow-Rosenheim reagent (mercuric acetate in nitric acid).

(b) Gives positive Tortelli-Jaffé reaction (specific reaction for ergosterol and its esters).

(c) Mixed M.P. with ergosteryl palmitate from P 75 showed no depression.

(d) The mycelium contained less than 0.1 % of free sterol precipitated by digitonin.

(e) Grown on Czapek-Dox medium and incubated at 30° for 37 days.

being calculated on the basis of the total mycelium from 90 flasks, i.e. from the metabolism of about 1575 g. glucose. The fat content of each mycelium was 2–3 % unless otherwise stated.

Investigation of the ergosteryl ester from the mycelium of P. brevi-compactum Dierckx (Cat. No. P 75).

The crude alcohol-insoluble product from about 2.5 kg. of ground mycelium (2.5 g.; M.P. 90–100°) was crystallised first from light petroleum (B.P. 60–80°; 40 cc.) then from acetone, and the purified product so obtained (0.5 g., M.P. 102–107°) was dissolved in hot benzene (30 cc.) and boiling acetone added until a turbidity appeared. The substance crystallised from the last-named mixture in colourless plates, M.P. 106–108° (0.4 g.). The melt became yellow at 150–160°, but solidified on cooling and remelted at 105–107°. The melting-point was not

altered by a further crystallisation from alcohol. The ester crystallised unchanged from 1 % alcoholic digitonin solution. It was also recovered unchanged when attempts were made to benzoylate it with benzoyl chloride in pyridine at 60–80° or to acetylate it by boiling with acetic anhydride.

Micro-analysis (Schoeller): C, 83.11, 83.20; H, 11.71, 11.63 %. $C_{44}H_{74}O_2$ requires C, 83.20; H, 11.76 %.

$[\alpha]_{5461}^{20} - 67^\circ$; $[\alpha]_{5790}^{20} - 52^\circ$. (0.0968 g. in chloroform (25 cc.) at 20° in a 2 dm. tube.) The chloroform solution turned yellow on standing for a day.

Molecular weight by hydrolysis. 0.4606 g. was boiled for 1½ hours with N/10 ethyl alcoholic potash (60 cc.) when it was found that a nett amount of 7.18 cc. N/10 alkali had been neutralised, corresponding to a molecular weight of 642 ($C_{44}H_{74}O_2$ requires mol. wt., 635). Ergosterol (M.P. 151–155°) was precipitated by adding much water to the strongly alkaline solution.

Isolation of ergosterol and palmitic acid by hydrolysis. 0.31 g. (M.P. 99–102°) was hydrolysed as above and the alcohol removed *in vacuo*. Water (50 cc.) and 2N NaOH (10 cc.) were then added, and the solution was extracted 3 times with much chloroform. The chloroform extract was dried over anhydrous sodium sulphate and evaporated, the solid residue being crystallised from alcohol to yield ergosterol (0.05 g.), M.P. 152–156°. The alkaline aqueous layer from which the chloroform had been separated was acidified with dilute sulphuric acid and extracted with light petroleum (B.P. 40–50°). The extract was dried over anhydrous sodium sulphate, evaporated and the residue crystallised from aqueous alcohol to yield a fatty acid (0.07 g.) melting at 53–57°. A further crystallisation from aqueous alcohol raised the M.P. to 55–59°. The mixed M.P. with an authentic specimen of palmitic acid (M.P. 62–63°) was 56–61°.

Micro-analysis (Schoeller): C, 74.83; H, 12.43 %. $C_{16}H_{32}O_2$ requires C, 74.91; H, 12.60 %; stearic acid (M.P. 69°), $C_{18}H_{36}O_2$ requires C, 75.98; H, 12.77 %.

Investigation of the ergosteryl ester from the mycelium of P. aurantio-griseum Dierckx var. Poznaniensis Zaleski (Cat. No. P 69).

The crude alcohol-insoluble product required less purification than the corresponding product from P 75. 1.1 g. was crystallised first from acetone (400 cc.) and then from alcohol (150 cc.). It formed shining plates, M.P. 103–106° (0.7 g.).

Formation of ergosterol and palmitic acid by hydrolysis. The above substance (0.66 g.) was hydrolysed by boiling N/10 alcoholic potash in the usual way, the alcohol removed, water and 10 cc. 2N NaOH added to the residue, which was then extracted 3 times with much chloroform. The product obtained by evaporation of the dried chloroform extract was crystallised once from alcohol to yield almost pure ergosterol (0.19 g.), M.P. 158–160°; $[\alpha]_{5461}^{24} - 160^\circ$; $[\alpha]_{5790}^{24} - 133^\circ$. Pure hydrated ergosterol has M.P. 160–163°, $[\alpha]_{5461}^{20} - 167.2^\circ$ [Callow, 1931].

The alkaline aqueous layer from which the chloroform layer had been partially separated was centrifuged until almost complete separation into two layers had taken place. The upper aqueous layer was then acidified with dilute sulphuric acid and the precipitated palmitic acid collected, well washed and dried (0.16 g.; M.P. 56.5–59°).

0.0893 g. in 25 cc. alcohol required 13.95 cc. N/40 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 256. $C_{16}H_{32}O_2$ (palmitic acid) has an equivalent of 256. The acid recovered from the above titration after crystallisation from aqueous alcohol had M.P. 57–61°. Found: C, 75.08; H, 12.58 %. $C_{16}H_{32}O_2$ requires C, 74.91; H, 12.60 %.

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A part of the crude acid (M.P. 56.5–59°, see above) was crystallised twice from light petroleum to yield a minute amount of colourless flakes, M.P. 61–62°. Mixed with authentic palmitic acid (M.P. 62–63°), the M.P. was 61–62°.

SUMMARY.

Ergosteryl palmitate has been isolated from the mycelia of 14 out of 15 species and strains in the *P. brevi-compactum* Dierckx series investigated, and also from the mycelium of *P. italicum* Wehmer, all the moulds having been grown on synthetic media. The mycelium of *P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski, grown on Raulin-Thom medium and incubated at 24° for 30 days, contains as much as 0.5 % of this ester.

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CLIX. THE OXIDATION OF SULPHYDRYL COMPOUNDS BY HYDROGEN PEROXIDE.

II. CATALYSIS OF OXIDATION OF CYSTEINE BY THIOCARBAMIDES AND THIOLGLYOXALINES.

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(Received July 3rd, 1933.)

THE first paper of this series [Pirie, 1931] dealt with the copper- and iron-catalysed oxidation of cysteine and glutathione by hydrogen peroxide in acid solution. It was shown that the corresponding disulphides were formed. Certain differences were observed between the kinetics of the copper-catalysed and the iron-catalysed reactions and no other metal of those tried was found to catalyse this type of oxidation. In the first paper brief mention was made of the fact that this action is catalysed by thiocarbamide; the present paper will be devoted to a study of this kind of catalysis and of the similar catalysis by chemically related substances.

EXPERIMENTAL.

The same polarimetric technique has been used in these experiments as in the series already published and the reagents, 1.0 % cysteine hydrochloride and 0.4 *N* hydrogen peroxide, were prepared in the same way.

Catalysis by thiocarbamide. "Metal-free" thiocarbamide can readily be prepared by crystallisation from dilute hydrochloric acid.

The kinetics of the thiocarbamide-catalysed oxidation resemble closely those of the copper-catalysed oxidation; the rate of oxidation, for example, is proportional to the concentrations of thiocarbamide and hydrogen peroxide but independent of that of cysteine hydrochloride. Curves illustrating these relations are given in Fig. 1.

In the paper already referred to it was shown that the iron-catalysed oxidation was inhibited by phosphate and pyrophosphate and the copper-catalysed oxidation, at p_H 4.6, by cyanide. These reagents have no effect on the thiocarbamide-catalysed oxidation; it seems therefore that this is not due to traces of metal impurity. Two further lines of argument are still more convincing. Firstly, the catalytic power is unaffected by repeated recrystallisation of the thiocarbamide; secondly, the residue obtained after incinerating 10–100 mg. of thiocarbamide with sulphuric acid, by the method already described [Pirie, 1931], is without catalytic activity. Curves 2 and 3 in Fig. 1 show that thiocarbamide has about the same catalytic power as 1/340 of its weight of copper.

I have been unable to demonstrate any effect of thiocarbamide on the aerobic oxidation of either cysteine or methylene blue at p_H 7.6. A few experiments have been carried out, with hydrogen peroxide, using glutathione instead of cysteine. In all respects these were analogous and the ratio of copper activity to thiocarbamide activity was once again 340.

There are two distinct products of the oxidation of thiocarbamide by hydrogen peroxide. Barnett [1910] prepared imino-aminomethanesulphinic acid, $\text{NH}:(\text{NH}_2)\text{CSO}_2\text{H}$, by oxidation in neutral solution, while Werner [1912]

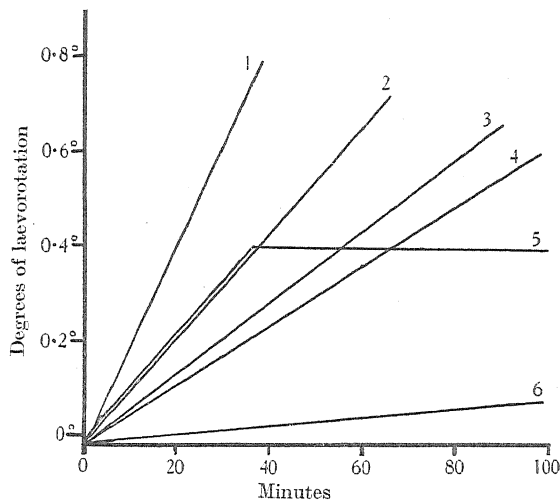


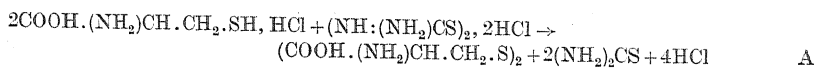
Fig. 1. Volume made up to 5 cc. in each case. Temperature 19° , p_{H} 2.1.

- | | |
|------------------------------|--|
| 1. 1 cc. 1 % cysteine-HCl; | 0.56 mg. thiocarbamide; 1 cc. 0.4 N H_2O_2 . |
| 2. " " | 0.28 mg. thiocarbamide; " " |
| 3. " " | 0.0005 mg. Cu " " |
| 4. " " | 0.28 mg. thiocarbamide; 0.5 cc. 0.4 N H_2O_2 . |
| 5. 0.5 cc. 1 % cysteine-HCl; | " " 1 cc. 0.4 N H_2O_2 . |
| 6. 1 cc. 1 % cysteine-HCl; | no catalyst; " " |

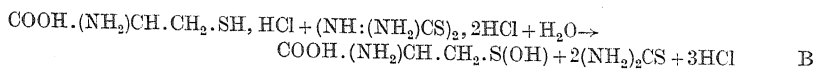
obtained salts of dithioformamidine, $\text{NH}:(\text{NH}_2).\text{CS}.\text{SC}:(\text{NH}_2):\text{NH}$, by oxidation in acid solution. The former has no catalytic power whereas the latter is both a catalyst and an oxidising agent.

Oxidation by dithioformamidine. Dithioformamidine dihydrochloride is readily prepared, following Werner's [1912] method, by the dropwise addition of twice the theoretical amount of sulphuryl chloride to a solution of thiocarbamide in alcohol. When washed with alcohol and ether and dried it seems to be stable indefinitely. The yield is 80 % of the theoretical.

In Fig. 2 the effect of adding the same amount of cysteine hydrochloride to varying amounts of dithioformamidine is illustrated. It is obvious that two distinct reactions can occur. The first is



while the second is most probably



To get clear-cut results it is essential to add the cysteine, with vigorous shaking, to the dithioformamidine; if the procedure is reversed some of the cysteine gets oxidised to cystine and this cannot be further oxidised at any appreciable rate. The product of reaction B, on the other hand, is almost instantly reduced to cystine by cysteine; this can easily be demonstrated by mixing half the quantity

of cysteine that was used in the experiments illustrated in Fig. 2 with one molecule of dithioformamidine, the other half of the cysteine is now added and, although reaction B takes place initially, the rotation is found to be 6.6° , *i.e.* the value corresponding to cystine. On standing for a few hours crystals of cystine separate from the reaction mixture. The cross in Fig. 2 represents the

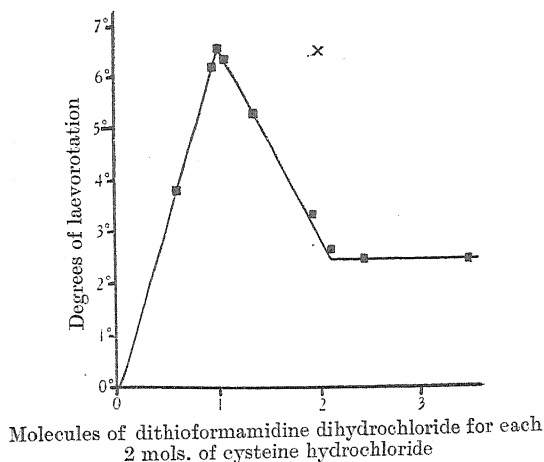
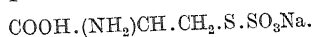


Fig. 2. 5 cc. of 3.12% (*M* 5) cysteine hydrochloride solution and the appropriate amount of dithioformamidine dihydrochloride solution used for each point. Volume made up to 10 cc. in each case.

rotation reached immediately when the usual quantity of cysteine hydrochloride is added to a molecule of dithioformamidine and the other molecule is added after 30 seconds. After several hours the rotation falls to the normal value for reaction B whereas the initial value is that for reaction A.

Similar results are obtained by use of the Folin and Marenzi [1929] method of cystine estimation. In this method sodium sulphite in alkaline solution is used to reduce the cystine to cysteine before the addition of the Folin uric acid reagent. Mirsky and Anson [1930] have pointed out that only half of the cystine is reduced under these conditions and Clarke [1932] has shown that the other half combines with the sulphite to form a cysteinesulphonate



It is interesting to note therefore that under these conditions no cysteine is formed from the product of reaction B. This compound, however, in the absence of sulphite, is very unstable in alkaline solution and reacts almost instantly to give a body that is estimated as cystine by the Folin-Marenzi technique¹. It seems therefore that the product of reaction B, apparently the sulphenic acid corresponding with cysteine, is stable only in acid solution whereas the complex obtained on the addition of sulphite is fairly stable in alkaline solution. This

¹ This method was used on account of its great convenience although it has frequently been shown to be by no means specific. I have, in fact, isolated from deaminated caseinogen [Dunn and Lewis, 1921] a sulphur-free compound, apparently a tyrosine derivative, which gives as much colour with the reagent as the same weight of cystine. This substance presumably accounts for the large amount of "cystine" found by Steudel and Schumann [1929] in deaminated caseinogen.

complex may be identical with that studied by Clarke. The preparation and properties of the sulphenic acid and of its alkaline decomposition products will be described fully in a later paper.

1 cc. of *M*/156 cysteine hydrochloride solution is added to each of seven flasks; these contain 0, 0.5, 0.7, 0.9, 1.0, 1.1 and 1.3 cc. of *M*/156 dithioformamidine dihydrochloride solution. The Folin-Marenzi reagents are now added, the sulphite being added first. The first flask gives twice as intense a colour as the second, the third and fourth are fainter while the fifth is very faint and the sixth and seventh give no more colour, at first, than the control. The sixth and seventh tend to darken on standing. If a large excess of dithioformamidine is added more colour develops and also a turbidity, due to the alkaline breakdown of the disulphide.

The evidence, both polarimetric and colorimetric, points therefore to a reaction of the type of B with the sulphenic acid as the most likely product. This compound has so far only been isolated in a crude form but it shows the expected properties.

Glutathione, like cysteine, reacts with dithioformamidine to give a product with a smaller rotation than oxidised glutathione.

Catalysis by compounds related to thiocarbamide. A number of compounds more or less closely related to thiocarbamide have been tried as catalysts for the oxidation of cysteine by hydrogen peroxide. These are listed in Table I.

Table I.

Substance	Method of preparation	Catalytic activity
Thiocarbamide	—	147 ± 7
<i>N</i> -Allylthiocarbamide	—	125
<i>N</i> -Phenylthiocarbamide	—	121
Ergothioneine	Pirie [1933]	126
<i>dl</i> -2-Thiolhistidine	Harington and Overhoff [1933]	127
2-Thiolglyoxaline	Marckwald [1892]	122
Ethylenethiocarbamide	Ruiz and Libenson [1930]	75
<i>N</i> -Phenyl-2-thiolglyoxaline	Wohl and Marckwald [1889]	60
<i>NN</i> -Methylphenylthiocarbamide	Gebhardt [1884, 1]	57
<i>N</i> -Ethyl- <i>N'</i> -phenylthiocarbamide	Weith [1875]	54
<i>NN</i> -Diethyl- <i>N'</i> -allylthiocarbamide	Gebhardt [1884, 2]	6.3
2-Thio-4-methylhydantoin	Johnson [1912]	10
<i>N</i> -Acetylthiocarbamide	Werner [1916]	3
<i>S</i> -Methyl- <i>pseudo</i> -thiocarbamide	Taylor [1917]	0
Diacetyl (<i>pseudo</i> ?) thiocarbamide	Werner [1916]	0
2-Thio-3-acetyl-4-methylhydantoin	Johnson [1912]	0
Thiobenzamide	Gabriel and Heymann [1890]	0

Also many other compounds such as methionine, *S*-ethyleysteine, ascorbic acid and urea are inactive.

The preparation of these compounds calls for little comment; in all cases the reactions described by the authors quoted were found to proceed smoothly and the substances were readily obtained pure. The pure substances were recrystallised from water or dilute alcohol, to which a little hydrochloric acid had been added, and were filtered off on an acid-washed glass funnel. Those substances which were catalytically active were carefully tested for metal impurity. The sample of *dl*-2-thiolhistidine used was given to me by Prof. C. R. Harington; it proved to be already free from catalytically active metals.

The figure in the third column of Table I is a measure of the catalytic power of each substance. Under the usual conditions, *i.e.* 1 cc. of 1 % cysteine

hydrochloride and 1 cc. of 0.4 *N* hydrogen peroxide made up to 5 cc., and measured in a 2 dm. tube, it is given by the expression

$$\frac{\text{Rate of change of observed rotation in degrees per hour}}{\text{Weight of catalyst in mg.}} \times \text{mol. wt. of catalyst.}$$

The conditions must be adhered to, for with these substances, as with thiocarbamide, the reaction velocity is proportional to the peroxide concentration as well as to the concentration of catalyst. It is independent of the concentration of cysteine. The temperature coefficient of the catalytic reaction is 2. Using this coefficient the values of the catalysis constant have been calculated for a temperature of 18°. The very marked differences between the constants for different substances will be discussed later.

The individual differences between the substances in Table I call for some comment. In two cases hydrogen peroxide acts on the catalyst in acid solution with the formation of colloidal sulphur. This action has, in the case of phenylthiocarbamide, been studied by Hector [1889; 1892] who showed that 3:5-diimino-2:4-diphenyl-1:2:4-thiodiazoltetrahydride was formed by the elimination of sulphur and hydrogen from two molecules of phenylthiocarbamide. In the presence of cysteine this action cannot apparently take place, but one observes colloidal sulphur in the polarimeter tube within a few minutes of the completion of the catalysed oxidation. *N*-Acetylthiocarbamide is broken down with the formation of sulphur at an early stage of the oxidation; polarimeter readings therefore are only possible for a few minutes with this catalyst. It is clear however that its catalytic power is very small. Hofmann and Gabriel [1892] have described a reaction with *NN*-methylphenylthiocarbamide very similar to that which occurs with phenylthiocarbamide. This action proceeds only in hot solution and I have not noticed the formation of colloidal sulphur under the conditions of these experiments.

Ergothioneine alone among the catalysts in Table I is optically active; this property allows one to study the ergothioneine-catalysed oxidation in some detail. The curves in Fig. 3 show the effect of adding various amounts of hydrogen peroxide to the same amount of ergothioneine. There are clearly two reactions. The first is between two molecules of ergothioneine and rather more than one molecule of hydrogen peroxide; presumably a disulphide is formed; Barger and Ewins [1911] have isolated the iodide of such a compound but I have not yet isolated the substance itself. This action is complicated by the more general breakdown, accompanied by desulphuration, which occurs with an excess of peroxide. As in the case of other thiolglyoxalines, sulphuric acid is formed. These two oxidations are catalysed by copper and iron but only to a very slight extent, *e.g.* 4% of copper (as CuSO_4) cause no appreciable shift of curve 3 in Fig. 3; with 40% however catalysis is noticeable. When studying the ergothioneine-catalysed oxidations it is important to use the minimum of ergothioneine so as to avoid the interference of its rotation with that of the cystine; it is also important to add the peroxide to the reaction mixture last; if it is added before the cysteine the ergothioneine will be partially desulphurised with loss of catalytic activity.

Catalysis by metals and thiocarbamides simultaneously. A few experiments have been made with copper or iron and thiocarbamide or ergothioneine acting together. When copper and iron are used together the rate of oxidation is the sum of the rates due to the two catalysts when acting independently; the same is true of thiocarbamide and ergothioneine. The catalytic effect of a mixture of copper and thiocarbamide is the sum of their independent effects for slow rates

of oxidation (0.0005 mg. Cu and 0.2 mg. thiocarbamide) only; when larger amounts of the catalysts are used the one is found not to increase the rate of oxidation due to the other; a similar state of affairs obtains with iron-thio-

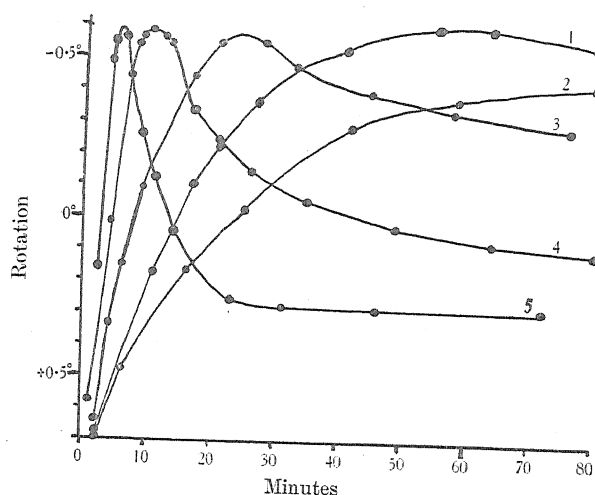


Fig. 3. Volume is 5 cc. in each case. Temperature 21°. 17.2 mg. of ergothioneine hydrochloride in each experiment.

1. 0.3 cc. of 0.4 N H_2O_2 .
2. 0.2 cc. "
3. 0.5 cc. "
- 0.5 cc. of 0.4 N H_2O_2 and 0.004 mg. Cu.
4. 1.0 cc. of 0.4 N H_2O_2 .
5. 2.0 cc. "

carbamide and metal-ergothioneine mixtures. In view of the readiness with which sulphur compounds form metal complexes these results are not wholly unexpected.

DISCUSSION.

The mechanism of this catalytic oxidation seems to be fairly simple. The results given in Fig. 3 together with the earlier work of Werner and others show that compounds of the type used in these experiments are readily oxidised in acid solution by hydrogen peroxide and that this oxidation does not need a metal catalyst. It is convenient to assume therefore that the first step is the formation of a disulphide and that this is immediately reduced by the cysteine. This step has only been followed in the case of the disulphide form of thiocarbamide, dithioformamidine. The formation of cyclic compounds, as in the case of phenylthiocarbamide, is probably irrelevant, for thiobenzamide behaves in this way [Hofmann and Gabriel, 1892] without being a catalyst.

It is clear from Table I that the more replaceable hydrogen atoms a thiocarbamide has the more powerful will be its catalytic effect. The thiolglyoxalines however, with two replaceable hydrogens, are comparable with the thiocarbamides with three, and phenylthiolglyoxaline with only one labile hydrogen is as catalytically active as a thiocarbamide with two. This enhanced activity is apparently due to the double bond, for ethylenethiocarbamide has about the activity

expected. The inactivity of the thiohydantoins is presumably due to the influence of the carbonyl group on the adjacent imino group; with two carbonyl groups the inactivity is complete. The very low value for the catalytic power of *N*-acetylthiocarbamide is however both interesting and unexpected.

Although ergothioneine occurs in blood corpuscles no function has yet been assigned to it: it is possible, though unlikely, that it acts normally as a catalyst in a manner similar to that in which it has been used in this work. The activity as an oxidising agent of the disulphide form of thiocarbamide, and also presumably of the disulphide form of ergothioneine, is however worthy of some attention. I have shown in this paper that it can oxidise cysteine to one of the many possible intermediate compounds between the cystine ingested by an animal and the sulphate excreted by it, and I hope later to describe further work on this type of oxidation.

Woodward and Fry [1932] and Benedict and Gottschall [1932] have found that, although ergothioneine is not readily oxidised by iodine, ferricyanide or arsenophosphotungstic acid in acid solution, it is oxidised by these reagents when in the presence of glutathione. With equal amounts of glutathione and ergothioneine only about 60 % of the ergothioneine is oxidised and with smaller amounts of glutathione the oxidation is very much smaller. I have tried small amounts (since both catalyst and oxidant are optically active it is difficult to interpret the results if large amounts are used) of cysteine hydrochloride (2 mg.) and glutathione (4 mg.) as catalysts for the hydrogen peroxide oxidation of ergothioneine, using the same conditions as in curve 3, Fig. 3. No catalytic effect could be demonstrated. With the exception of the work just referred to no catalysed oxidation in any way similar to that described in this paper seems to have been studied. The resemblances between this type of oxidation and an enzyme action are interesting but need not, at the present stage, be emphasised. Bersin [1932] has described the effect of a large group of substances on the autoxidation of thiolacetic acid anilide in alkaline alcoholic solution. The active substances include iodoacetic acid, creatine and thianthrene, and it is hard to see any sign of a possible common action; moreover the shapes of the curves published make it probable that some of these substances catalyse different reactions. Bersin explains some of his results by the intermediate formation of thio-ethers. I have tried methionine and ethylcysteine as catalysts for the hydrogen peroxide oxidation of cysteine and have found them quite inactive.

SUMMARY.

Thiocarbamide catalyses the oxidation of cysteine to cystine by hydrogen peroxide in acid solution.

Dithioformamidine can oxidise cysteine to a sulphenic acid; this is reduced to cystine by cysteine. Dithioformamidine has no effect on cystine.

Substituted thiocarbamides and related substances, such as ergothioneine, are catalytically active and a theory for the mechanism of the action has been put forward.

Glutathione behaves throughout in a manner similar to cysteine.

I am very grateful to Sir F. G. Hopkins for the interest he has taken in this work.

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CLX. A STUDY OF SEASONAL VARIATION IN BUTTER-FAT.

I. SEASONAL VARIATIONS IN CAROTENE, VITAMIN A AND THE ANTIMONY TRICHLORIDE REACTION.

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(Received July 5th, 1933.)

IN January 1932 work was started in the National Institute for Research in Dairying to investigate the variations in the vitamin content of typical English milk as determined by the season of the year and the nutritional condition of the cow. This work, which was planned to cover two complete years, and which will be described in detail later, is mainly concerned with the accumulation of data obtained in routine biological tests carried out according to accepted methods. In several instances however unexpected difficulties have been encountered, and it has been necessary to seek new explanations of the observed facts. Among other occasions this was the case when it was attempted to apply the well-known colorimetric and spectroscopic methods to the estimation of carotene and vitamin A in butter-fats, and the present joint work with the Nutritional Laboratory is concerned with our attempts to overcome this difficulty.

In assaying the vitamin A of cod-liver oils by the antimony trichloride method it is well known that the blue value calculated per unit of the original oil may be greatly increased as the result of saponification. Green [1932] and Moore [1932] have already noticed that the blue value given by butter-fat is similarly increased on saponification, and this finding has been confirmed in the National Institute for Research in Dairying in routine tests on over 150 samples of butter-fat. What specially attracted our interest however was the fact that the blue value was not always increased in the same ratio, and that in general the increase was much greater in samples of butter obtained from cows at pasture ("summer butters") than in samples obtained from stall-fed cows receiving diets totally or partially deficient in green fodder ("winter butters"). In addition it was noticed that the colours produced by the untreated winter and summer butter-fats differed qualitatively. The maximum intensity of blue in the case of winter butter was given immediately on the addition of the antimony trichloride reagent, after which fading took place very rapidly (2-5 secs.) without the formation of any intermediate tint. With summer butters on the other hand the blue colour was usually more stable, sometimes taking a few seconds to reach its maximum value, while fading was preceded by the change in the colour of the solution from the original greenish-blue to a dull reddish-blue. Now it is well known that the blue colour given by vitamin A in the antimony trichloride test is "inhibited," a term which may be considered to embrace diminution in intensity, alteration in tint or acceleration in the

rate of fading, by a wide variety of substances [Corbet *et al.*, 1933]¹. Thus unsaturated acids and oils cause inhibition when present in relatively high concentration [Norris and Church, 1930], while substances of the indole type [Emmerie *et al.*, 1931] and also a highly unsaturated fraction obtained from the acids derived from cod-liver oil [Emmerie, 1933] appear to act more specifically and cause inhibition when present in very small amounts. In a preliminary communication [Booth *et al.*, 1933] we have already suggested that the essential cause of the different behaviour of untreated summer and winter butter-fats in the antimony trichloride reaction lies in the fact that not only the vitamin A content, but also the inhibitory power of the fat is increased in summer butters. We have also pointed out that the free fatty acids prepared from summer butter differ from those from winter butter in showing much stronger absorption at 230 $m\mu$, and much more rapid brown colour formation when treated with the antimony trichloride reagent. Since however it is not yet clear whether these two properties are genuinely related to the increased inhibitory power of summer butter-fat we shall not include data obtained on the free fatty acids in the present paper but shall confine ourselves to a description of inhibitory power only in so far as it affects the colorimetric estimation of the carotene and vitamin A.

EXPERIMENTAL.

The 15 butter-fats examined in the present work were selected from a large collection of samples which had been accumulated and examined in the course of about 13 months as the result of bi-weekly collections of milk from the typical herd of Shorthorn cows kept at Shinfield. The butter was churned in the Institute's experimental dairy and was unsalted. It was rendered without delay. When not in use the butter-fats were stored in a refrigerator. No noticeable deterioration occurred under these conditions.

Carotene estimations. There is little evidence that any pigment other than β -carotene is normally present in significant amounts in butter-fat, and in the present work the intensity of yellow pigmentation has been taken as a measure of the carotene concentration. The yellow units per g. of butter-fat were measured in a Lovibond tintometer using a cell of such thickness as to give a reading of 2–5 Y. This reading was then corrected for thickness of the cell and for the difference of the specific gravity of butter-fat from unity^{2,3}.

¹ Interference in the application of the $SbCl_3$ reaction may also arise through clouding of the reagent (as by water, alkali, medicinal paraffin or squalene), or through the development of an intense colour by the substance added which masks the vitamin A blue (as in the case of the brown colour formed by large amounts of unsaturated acids, probably through impurity, or by rancid fats). These forms of interference can hardly be considered to represent true "inhibition."

² The accuracy of observation is limited by two factors. On the one hand the matching of shades of more than 10 Y is very difficult, involving a great personal variation according to the amount of red introduced. On the other it is found that the readings obtained on using cells of different thickness (1 cm., $\frac{1}{2}$ cm., $\frac{1}{4}$ cm.) do not show a linear relationship. The same effect is observed when the thickness of the cell is kept constant, and the fat diluted with a suitable solvent. The plan of keeping the reading within defined limits, as described above, seemed to be least objectionable and was always adopted except in early experiments carried out before a $\frac{1}{4}$ cm. cell had been acquired. In these cases readings of the order of 10 Y, taken in a $\frac{1}{2}$ cm. cell, were subsequently corrected to allow for the slightly lower reading that would have been obtained if a $\frac{1}{4}$ cm. cell had been used.

³ In earlier experiments yellow values were estimated both on the untreated butter-fat and on the non-saponifiable residue. The values obtained in the latter case, however, always indicated a quantitative transference of pigmentation to the non-saponifiable residue, and observations on the latter therefore appeared to be unnecessary. In the present paper data obtained using the untreated butter-fat only are given.

Example. Sample in $\frac{1}{2}$ cm. cell gave reading of 3.2 Y.

$$\text{Y.U. per g.} = 3.2 \times 2 \times \frac{1}{0.9} = 7.1.$$

Blue colour estimation. Untreated butter-fat. The blue values of the untreated butter-fats were estimated by treating 0.5 g. of the melted fat with 2 cc. of the SbCl_3 reagent in a 1 cm. cell, and observing the maximum blue reading attained. Since the colours given were extremely transient, especially in the case of winter butters, observations could only be carried out by setting the tintometer at the expected reading as indicated by preliminary trials, and then, after rapidly adding the reagent to the fat, noticing whether the maximum colour produced was more or less than the expected reading. Through practice fairly consistent results could be obtained in this way.

Blue colour estimation. Non-saponifiable residue. Saponification of the butter-fats was effected by boiling together 5 g. of the fat, 2.2 cc. of a saturated solution of KOH in distilled water and 10 cc. of ethyl alcohol. In earlier experiments boiling was continued for 1 hour under a reflux condenser, but this procedure was subsequently modified since saponification was found to be complete after boiling for 2 minutes. The alteration in the time of boiling had no effect on the blue value of the non-saponifiable residue, and it will therefore be unnecessary to indicate which procedure was adopted in individual samples. To obtain the non-saponifiable residue the soaps were diluted with 40 cc. of water and extracted with 3 portions of 50, 25 and 25 cc. of ether. The extracts were combined, washed with water, dried with anhydrous sodium sulphate and evaporated on a water-bath, the last traces of solvent being removed under diminished pressure.

The non-saponifiable residue so obtained was dissolved in 5 or 10 cc. of chloroform. An amount of this solution expected to give a blue reading of 3-5 was made up to 0.5 cc. with chloroform and treated in a 1 cm. cell with 2 cc. of the antimony trichloride reagent. The colour produced did not fade so rapidly as in the case of the untreated butter-fats, but to obtain maximum values it was necessary in some cases to take readings rapidly. Results were calculated as follows.

Examples. Untreated butter.

0.5 g. with 2 cc. SbCl_3 reagent gave 2 B 0.5 Y.

$$\begin{aligned} \text{B.U. per g.} &= 2 \times 2.5 \text{ (total vol. of mixed solutions)} \times \frac{1}{0.5} \\ &= 10 \text{ B.U. per g.} \end{aligned}$$

Non-saponifiable residue from 5 g. butter-fat was dissolved in 10 cc. CHCl_3 . 0.3 cc. of this solution with 0.2 cc. CHCl_3 + 2 cc. SbCl_3 gave 4 B 1 Y.

$$\begin{aligned} \text{B.U. per g. butter-fat} &= \frac{4 \times 2.5 \times 10}{5 \times 0.3} \\ &= 67. \end{aligned}$$

The above colorimetric methods, of course, do not permit a high degree of accuracy. Personal errors in matching the colours are difficult to exclude, particularly when a high proportion of a second colour (*e.g.* yellow in the case of the SbCl_3 blue) or the use of neutral glasses is necessary to obtain the correct shade. Moreover the conditions of illumination of the tintometer, which in the present instance were quite different at Shinfield (artificial light) and Cambridge (diffuse reflected daylight), must be taken into account. To avoid variations arising from errors of the above type the results obtained in the two laboratories were not intermixed, but each series was taken from results obtained in one laboratory. In most cases the general conclusions reached were confirmed by parallel work in the other laboratory.

Table I.

Date	Yellow value (Y.U. per g.) Untreated butter-fat	Blue value (B.U. per g.)		Ratio blue value N.S.R. blue value fat
		Untreated butter-fat	Non- saponifiable matter	
1932 April 26	2.4	8	16	2
Cows went out to pasture April 26th.				
May 3-4	10.5	10.5	66	6
May 10-16	17.5	7	66	9
June 22-28	23	10	63	6
July 12-Aug. 2	17.5	7	62	9
Sept. 30-Oct. 13	19.4	8	67	8
Oct. 31-Nov. 3	16.5	7	72	10
1933 Jan. 26-30	2.6	7	42	6
Feb. 24-27	2.8	12.5	35	3
March 24-27	3.0	13	40	3
April 14-17	2.3	12	34	3
April 19-20	3.2	13	36	3
Cows went out to pasture April 20th.				
April 27-28	7.2	9	64	7
May 9-11	13	8	68	8.5
May 19-22	18	7.5	72	10

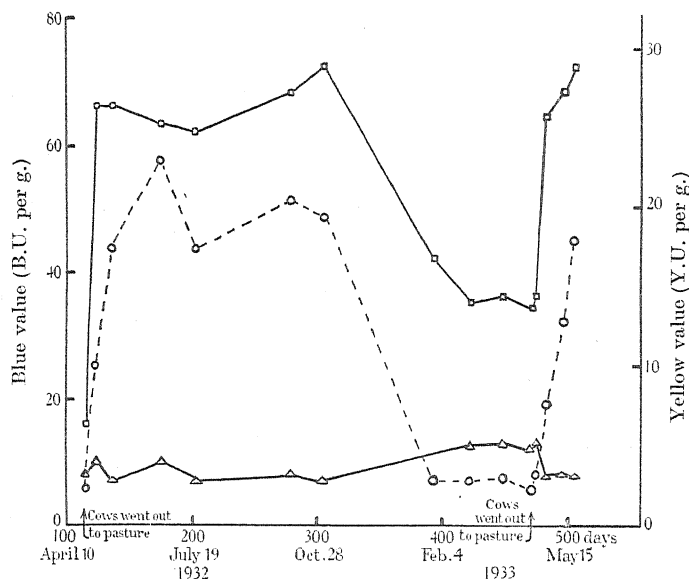


Fig. 1. Seasonal variations in butter-fat. The upper continuous curve indicates the SbCl_5 blue value determined on the non-saponifiable residue, the lower continuous curve the blue value as determined on the untreated butter-fat. The broken curve indicates carotene expressed as yellow units determined on the untreated fat. The blue and yellow scales have been adjusted on the assumption that 2 Y.U. of carotene are equivalent biologically to about 5 B.U. of vitamin A.

Seasonal variation in carotene content. The seasonal variation in carotene content as indicated by the yellow value is given in Table I and shown graphically in Fig. 1. The first value given relates to a very faintly coloured butter-fat obtained during the last weeks of stall feeding in April 1932. In agreement with

well established precedent there was a sharp rise in the carotene content coinciding with the turning out of the cows to pasture, after which the value remained almost constant until the autumn, when a gradual fall began which continued throughout the winter, during which, of course, stall feeding was adopted with an increasing scarcity of green fodder. The butters of March and April 1933 were again very faintly coloured, and the rise in carotene content was repeated on turning out the cows to pasture as in the previous year.

Seasonal variation in the blue value of the untreated butter-fat. Data are given in Table I and Fig. 1. The value varied between comparatively narrow limits (7-13 B.U. per g.) and was usually somewhat greater in "winter" than in "summer" butter-fats.

Seasonal variation in the blue value of the non-saponifiable residue (see Table I and Fig. 1). In contrast with the case of the untreated butter-fat, the blue value of the non-saponifiable residue showed a marked seasonal variation. The value rose sharply when the cows were turned out to pasture in the spring of 1932, remained constant during the summer and autumn, declined during the winter and rose again when the cows were turned out to pasture at the end of April 1933. The variation thus followed the carotene content, although the disparity between the winter minimum and summer maximum was not so great.

Seasonal variation in the ratio of the blue values as determined on the non-saponifiable residue and on the untreated butter-fat. In the above experiments the blue value per g. of butter-fat was invariably greater when the determination was carried out on the non-saponifiable residue than when the butter-fat was treated directly with the SbCl_3 reagent. It will be seen from Table I, however, that the ratio of the two values was not constant, but was generally greater in summer than in winter samples.

Seasonal variation in the inhibitory power of the butter-fat in the SbCl_3 reaction of vitamin A. The inhibitory powers of the winter and summer butter-fats were compared by observing the reduction in blue reading caused by the addition of a fixed amount of the butter-fat to a fixed amount of vitamin A concentrate (B.D.H.) before treatment with the SbCl_3 reagent. It must be emphasised that both winter and summer butter-fats interfered in the SbCl_3 reaction of vitamin A, although in different ways. With winter butter-fat the first flash of colour produced approximated to that due to the added concentrate, after which rapid fading took place. With summer butter, on the other hand, the first flash of colour was completely inhibited, and instead the much less intense dull blue which appears to be given by the vitamin in the presence of inhibitory substances gradually developed. By taking readings immediately after the addition of the reagent, therefore, a decided seasonal difference could be demonstrated.

In Table II data are given showing the effect of adding 0.4 cc. of butter to 0.0055 mg. of a vitamin A concentrate, which by itself gave a reading of 7 B.U. when dissolved in 0.5 cc. of chloroform and treated with 2 cc. of the SbCl_3 reagent. The colour readings observed immediately after adding the reagent and also after standing for 30 seconds are given. In Fig. 2 these results are shown graphically. The inhibitory power shows a seasonal variation similar to that already shown to occur in the carotene and vitamin A contents¹.

¹ In the above experiments the blue colour observed must have been derived not only from the concentrate but also from the butter added as inhibitor. The amount of vitamin present in summer fats was, of course, much more than in winter fats. The degree of inhibition by summer fats was therefore even greater than would appear at first sight.

Table II.

		Immediate blue reading	Blue reading after 30 secs.
Concentrate with 0.4 cc. of butter fat	1932 May 3-4	6	3
	May 10-16	3.5	3
	June 28	3	3
	July 12-Aug. 2	2.5	2.5
	Sept. 30-Oct. 13	2.5	2.7
	Oct. 31-Nov. 3	3.5	3.0
	1933 Jan. 26-30	5.5	2.5
	Feb. 24-27	6	2
	March 24-27	6	2
	April 19-20	6	2.5
Concentrate alone	April 27-28	4	2.5
	May 9-11	3	2.5
		7	6.5

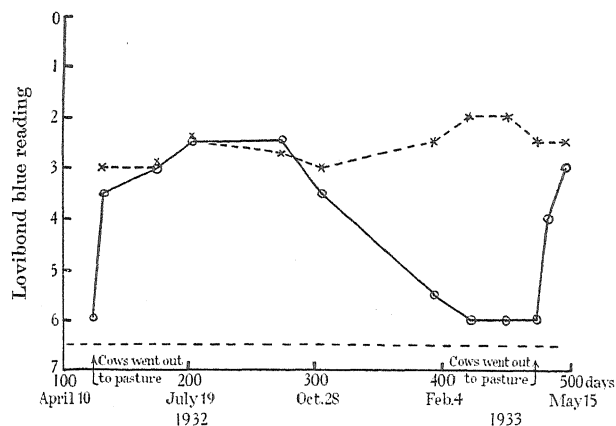


Fig. 2. Seasonal variation in the inhibitory power of butter-fat on the SbCl_3 reaction of added vitamin A concentrate. The continuous curve gives readings immediately after adding reagent, the broken curve gives readings after 30 secs. The concentrate by itself gave a reading represented by the horizontal axis; after 30 secs. the colour faded to correspond with the dotted line just above the horizontal axis. The scale of Lovibond blue readings has been inverted so that the curve rises as the inhibitory power of the butter-fat increases.

DISCUSSION.

The above data on the yellow value of untreated butter-fat and on the blue value of the non-saponifiable residue provide additional confirmation of the already well established seasonal variation in the carotene and vitamin A contents of butter-fat depending on the change in the nutrition of the cow on turning out to grass.

On the other hand the blue value of the untreated butter-fat is almost valueless as a guide to vitamin A content. In this case the intensity of blue colour given by the butter-fat is affected in a positive sense by the amount of vitamin A present, but also in a negative sense by the inhibitory power of the fat. The effect of pasture feeding is to increase both these variables, with the result that the effects cancel out and the observed blue value is little changed¹.

¹ The blue value of the untreated butter-fat may sometimes show a definite increase under abnormal conditions of feeding. Thus Moore [1932] found the blue value to be increased on supplementing the stall dietary with large amounts of carrots. A similar result may be obtained by feeding cod-liver oil.

Apart from the question of following the seasonal variation, the blue value determined on the untreated fat in any case represents only 1/10-1/5 of the true blue value as determined on the non-saponifiable residue.

In regard to the practical problem of assaying the total vitamin A activity of butter-fats by colorimetric methods it is obvious that both carotene and preformed vitamin A must be taken into account. The carotene content, on the assumption that yellow colour is entirely due to this pigment, may be calculated on the basis that pure carotene has a yellow value of about 2000 Y.U. per mg. The vitamin A content, to avoid unnecessary assumptions as to the blue value of pure vitamin A, may be expressed in blue units determined on the non-saponifiable residue¹. On attempting to combine the carotene and vitamin A contents to arrive at the total activity of the butter-fat, however, we are faced with the difficulty that no general agreement has yet been reached on the relative activities of carotene and vitamin A. To help in the interpretation of the present results, and without implying that an accurate ratio has yet been determined, it will be assumed from the recent work of Moore [1933] that 2 Y.U. of carotene are roughly equivalent to 5 B.U. of vitamin A. Acting on this assumption, and for purposes of simplicity accepting the international standard of vitamin A activity as referring to 1 γ of pure carotene², the total activity of a given sample of butter-fat might be calculated as follows.

$$\frac{\text{Y.U. per g.}}{2} + \frac{\text{B.U. (N.S.R.)}}{5} = \text{international units per g.}$$

When we apply this formula to typical samples of "winter" (April 19-20, 1933, giving 3.2 Y.U., 36 B.U. per g.) and "summer" (May 19-22, 1933, giving 18 Y.U., 72 B.U. per g.) butter-fats it will be seen that the total vitamin A value is increased from about 8.8 to about 23.4 units per g. The contribution of carotene to the total activity is of course much more substantial in the "summer" sample, representing about 1/3 of the total activity. Biological experiments to decide the actual distribution of activity between carotene and vitamin A in summer butters by means of tests on untreated butter-fat and the same sample after decoloration by treatment with charcoal are now being carried out by two of us (R. G. B. and S. K. K.).

In a subsequent communication we hope to deal with spectroscopic variations in winter and summer butter-fats.

SUMMARY.

1. The seasonal variation in the carotene and vitamin A contents of typical English butter from Shorthorn cows was followed by colorimetric methods. Determinations of the intensity of yellow colour were carried out on the untreated butter-fats. SbCl₃ blue values were determined both on the untreated fats and the corresponding non-saponifiable residues.

¹ Theoretically a correction equal to about 1/10 of the yellow value should be deducted from the blue value of the non-saponifiable residue to account for the amount of blue colour contributed by the carotene present. This correction, however, would appear to be so small as to represent an unnecessary refinement at the present stage of accuracy. In agreement with this view, in the case of most butters very little difference could usually be detected in the blue value of the non-saponifiable residue when determined on the natural yellow fat and on the same sample saponified after the removal of the carotene by absorption on charcoal.

² The international standard carotene is admittedly not of the highest attainable purity, but here again it is obviously premature to suggest a correction until general agreement has been reached.

2. Data obtained on yellow colour and blue value as determined on the non-saponifiable residue were in good agreement with the well established variation of the carotene and vitamin A contents of the butter corresponding with the quantity of grass or of green fodder available in the diet of the cow.

3. Blue values determined on the untreated butter-fat were found to be valueless as a guide to vitamin A content, being 5-10 times lower than values determined on the non-saponifiable matter.

4. Although blue values determined on the untreated fats showed no consistent quantitative variation, qualitative differences in the behaviour of the blue colours produced by "winter" and "summer" butter-fats were observed. These differences were connected with an inhibitory substance or substances, which was present in much larger concentration in "summer" than in "winter" butter-fats, and whose presence could be detected from its inhibitory power on the blue colour given by vitamin A when added from an external source.

5. In the practical assay of the total vitamin A activity of butter-fats by colorimetric means it is necessary that both the vitamin A and carotene contents should be taken into account. A provisional formula for calculating the total vitamin A activity is suggested. The total vitamin A activity of summer butter-fat from Shorthorn cows appears to be some three times greater than that of winter butter-fat. The fraction of the total activity due to carotene is also greater in summer butter-fat.

Our thanks are due to Capt. J. Golding, Dr L. J. Harris and Prof. H. D. Kay for their valuable criticisms, and to Miss D. V. Dearden for the churning of the butters.

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CLXI. THE BIOCHEMISTRY AND PHYSIOLOGY OF GLUCURONIC ACID.

I. THE STRUCTURE OF GLUCURONIC ACID OF ANIMAL ORIGIN.

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(Received May 31st, 1933.)

THE work described in this communication records the successful application to bornyl-*d*-glucuronide (borneolglucuronic acid) of the standard methods of elucidating the ring structure of sugar derivatives. Bornylglucuronide was isolated by Clement and Fromm [1902] from the urine of rabbits fed on borneol, and later Quick [1927] prepared it from the urine of dogs similarly fed. A full description of the general properties of this compound, however, was not given. Its formation was also detected by Hildebrandt [1909] after subcutaneous injection of a solution of bornylglucoside in rabbits. In identification Hildebrandt quotes only the melting-point, 174°. No previous investigation of the structure of a glucuronide synthesised in the animal body has been described. Whilst this study was in progress, two papers appeared dealing with the structure of uronic acid residues in naturally occurring products of plant origin. An aldobionic acid from gum arabic has been the subject of a study by Challinor, Haworth and Hirst [1931]. By methylation and hydrolysis they find it to be a 6-galactopyranose-*d*-glucuronic acid. The glucuronic acid residue was isolated, after hydrolysis of the methylated aldobionic acid, as syrupy trimethylglucuronic acid, which on methylation with methyl sulphate and alkali gave crystalline trimethyl- β -methylglucuronide. Then, by comparison of the rate of hydrolysis of the latter with that of β -methylglucopyranoside, these authors deduced a pyranoid structure for the glucuronide. Furthermore, Robertson and Waters [1931] have studied the structure of euxanthic acid, a compound of euxanthone and glucuronic acid. Euxanthic acid was methylated and the product hydrolysed. From the products of hydrolysis trimethylglucuronic acid [*cf.* Challinor *et al.*, 1931] was isolated. This was oxidised with nitric acid, and the products of oxidation, after esterification and distillation, yielded dimethyl *d*-dimethoxy-succinate and 2:3:4-trimethyl- δ -saccharolactone methyl ester. The saccharolactone was then prepared independently from a compound of known pyranoid structure, 2:3:4-trimethyl- α -methylglucoside, and hence its isolation from the oxidation products of trimethylglucuronic acid establishes a pyranoid structure for the glucuronic acid residue of euxanthic acid. It may be mentioned here that Robertson and Waters did not find *i*-xylotrimethoxyglutaric acid in the products of oxidation.

In the work here described it is proved by direct chemical evidence that the glucuronic acid residue of bornylglucuronide synthesised in the dog is a pyranoid compound. β -Bornyl-*d*-glucuronide was methylated with methyl iodide and silver oxide to give the methyl ester of 2:3:4-trimethyl- β -bornyl-*d*-glucuronide which was isolated as a white crystalline solid, m.p. 92–93° and $[\alpha]_{5461} - 30.7^\circ$

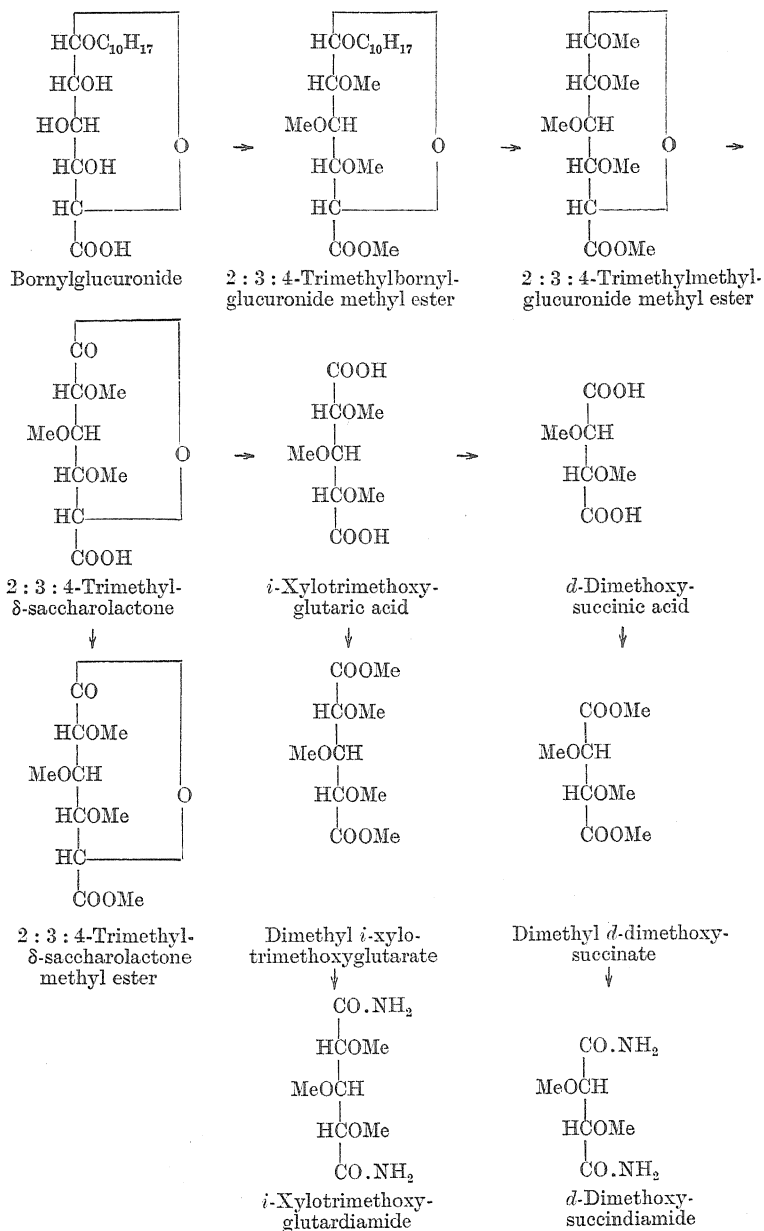
in alcohol. This ester was then subjected to the action of 0.2*N* sulphuric acid in methyl alcohol at 100° for 24 hours in a sealed tube, and a liquid mixture of the α - and β -isomerides of the methyl ester of 2:3:4-trimethylmethylglucuronide was isolated and purified by distillation in a high vacuum. This ester had $[\alpha]_{5461} + 98.9^\circ$ in water. The fully methylated glucuronide was then carefully oxidised with nitric acid (sp. gr. 1.42) for 2½ hours, the temperature being regulated and never allowed to rise above 90°. After removal of the bulk of the nitric acid by continuous distillation with water, the products of oxidation were esterified with 3 % hydrogen chloride in methyl alcohol. After neutralising the acid with silver carbonate, the solvent was evaporated and the syrupy residue distilled in a high vacuum and fractionated.

The first and second fractions, from their rotations in methyl alcohol, were found to be mixtures of about 20 % of dimethyl *d*-dimethoxysuccinate and 80 % dimethyl *i*-xylotrimethoxyglutarate [see Hirst, 1926; Hirst and Purves, 1923]. The third fraction consisted mainly of 2:3:4-trimethyl- δ -saccharolactone methyl ester [Robertson and Waters, 1931]. On treatment of fractions 1 and 2 with ammonia in methyl alcohol at 0°, each gave two separate crops of crystals on standing. The first of these was collected after 2 days' standing and when purified was identified as *d*-dimethoxysuccindiamide by analysis and by comparison of the constants with those given by Haworth, Hirst and Miller [1927] for this compound. The second crystalline deposit was collected over a period of 14 days after the separation of the first crop and was identified as *i*-xylotrimethoxyglutardiamide by analysis and comparison with the constants given for this compound by Hirst and Purves [1923].

After 4 days' standing in a desiccator, the third fraction of the products of high vacuum distillation partially crystallised. On extracting the partially crystalline mass with small quantities of ether, the syrupy part was removed leaving a white crystalline solid which was only slightly soluble in ether. These crystals (platelets) were then identified as 2:3:4-trimethyl- δ -saccharolactone methyl ester by analysis, rotation, melting-point and titration; this lactone ester was described for the first time by Robertson and Waters [1931] and shown to possess a pyranoid structure.

The isolation of *i*-xylotrimethoxyglutaric acid derivatives from the products of oxidation can only be explained by ascribing to the original glucuronide a pyranoid structure, since carbon atoms 2, 3 and 4, with their attached methoxy-groups, have been isolated as crystalline *i*-xylotrimethoxyglutardiamide. The *d*-dimethoxysuccinic acid derivatives which were obtained in small yield are further oxidation products of the *i*-xylotrimethoxyglutaric acid. Additional evidence for the pyranoid structure of the uronic acid residue of bornylglucuronide is obtained from the isolation of the crystalline 2:3:4-trimethyl- δ -saccharolactone methyl ester, which, as has already been mentioned, is known to possess a pyranoid structure. The arguments therefore prove that bornylglucuronide has a similar structure to the normal glucosides and that the uronic acid residue follows the parent hexose, glucose, in possessing a pyranoid structure. The appended sequence of structural formulae (p. 1199) illustrates the above argument.

It will be observed from the following formulae that any trimethoxyglutaric acid formed by oxidation of glucuronic acid should be optically inactive since its stereochemical arrangement would give internal compensation; on the other hand, the dimethoxysuccinic acid formed by further oxidation, not being internally compensated, should be optically active. The experimental results are in conformity with these expectations.



EXPERIMENTAL.

Administration of borneol. Isolation of zinc bornylglucuronide.

3 to 4 g. of borneol¹ were fed daily to each of several dogs weighing from 20 to 30 lbs. Initially the borneol was fed smeared on meat, but since the dogs

¹ Borneol (Harrington's) $[\alpha]_{5461}^{+20}$ (in EtOH).
Borneol (B.D.H.) $[\alpha]_{5461}^{+26.5}$ (in EtOH).

finally refused to eat any food tasting of borneol it was found easy and advantageous to administer it in small gelatin capsules. The capsules could be concealed inside pieces of meat which were rapidly swallowed by the dogs. Each capsule held about 0.5 g. of borneol. The dogs were kept in a large zinc-lined cage, which carried under the floor a large shallow zinc funnel, so that all the urine excreted could be collected in a beaker placed at the lower orifice of the funnel. The urine collected was worked up daily by a method based upon that of Quick [1927]. The urine was acidified with acetic acid and precipitated with the exact quantity of normal saturated lead acetate and filtered. The clear yellow filtrate was heated to the boiling-point and about 10 g. of solid zinc acetate were added to every 500 cc. of hot filtrate. The vessel was well shaken to initiate the separation of the zinc salt and, after standing for about 2 minutes, it was filtered under suction, washed with warm water and dried in the air. With stale urine the yield of zinc salt was low, and it had a brown colour and urinary odour. The salt develops a brown colour and urinary odour if, after precipitation, it is allowed to stand in contact with the mother-liquor for some time before filtering. Otherwise the salt is pure white and odourless. The yield was 0.5 to 0.6 g. per g. of borneol fed. The salt is insoluble in water and ordinary organic solvents, but is soluble with hydrolysis in dilute acids. This salt was first prepared by Clement and Fromm [1902] who gave it the formula $C_{32}H_{50}O_{14}Zn, 2H_2O$. In the present investigation it was prepared for analysis by boiling with distilled water, filtering under suction, washing with water, alcohol and ether and drying in a desiccator. It was then analysed for zinc by the zinc ammonium phosphate method. Found Zn, 8.56 %; $C_{32}H_{50}O_{14}Zn, 2H_2O$ requires Zn, 8.61 %.

Only a few experiments were carried out on human subjects, for reasons which will be dealt with in a later paper, but the zinc salt was isolated in the same way as for dogs and in about the same yield.

The preparation of β -bornyl-d-glucuronide.

The powdered zinc salt (50 g.) was dissolved in 80 cc. of hot 3.5N sulphuric acid and the solution filtered while hot on a glass filter. The filtrate was then allowed to stand in the refrigerator overnight whereby it became a semi-solid slightly brown crystalline mass. The liquid was filtered under suction and the solid washed with ice-cold water. It was recrystallised from hot water, decolorising with charcoal. The yield was 35 to 40 g. For titrations and optical rotations some specimens were recrystallised several times from hot water. β -Bornyl-d-glucuronide forms microscopic prismatic needles similar to those described by Clement and Fromm [1902] for the corresponding menthylglucuronide. It is odourless when pure and is easily soluble in hot water, ether and alcohol, slightly soluble in cold water, and practically insoluble in chloroform. Contrary to Quick [1927], who states that bornylglucuronide possesses water of crystallisation approximating to one molecule, the titrations described below show it to contain exactly 1.5 molecules of water; it is thus similar to the corresponding menthylglucuronide which also possesses 1.5 molecules. It melts at 174–175°. Its specific optical rotation will be dealt with in a later publication.

Titration (microburette).

Several titrations were carried out with highly purified specimens.

1. Air-dried specimen.

0.1010 g. required 5.13 cc. of 0.055N NaOH.

5.14 cc. is required for $C_{16}H_{26}O_7 + 1.5H_2O$,

5.28 cc. is required for $C_{16}H_{26}O_7 + H_2O$.

2. *Dried over sulphuric acid.*0.1453 g. required 6.55 cc. of 0.0625 *N* NaOH.6.51 cc. is required for $C_{16}H_{26}O_7 + 1.5H_2O$,6.75 cc. is required for $C_{16}H_{26}O_7 + H_2O$.3. *Dried at 137° over P_2O_5 in vacuo.*0.1009 g. required 4.91 cc. of 0.0625 *N* NaOH.4.89 cc. is required for $C_{16}H_{26}O_7$ (*i.e.* anhydrous).*The methylation of β -bornyl-d-glucuronide.**Isolation of 2:3:4-trimethyl- β -bornyl-d-glucuronide.*

Bornylglucuronide (3.3 g.) was methylated with silver oxide and methyl iodide in the usual manner. The reaction at first proceeded vigorously in the cold owing to esterification of the free carboxyl group, and later the methylation was continued at 45 to 50°. Four separate treatments with fresh silver oxide and methyl iodide were necessary to methylate the compound completely, and the methoxyl content of the product increased as follows: syrup after the second methylation had OMe, 25.46 %; after third methylation, OMe, 29.61 %; after fourth methylation, OMe, 30.63 %. The product of the fourth methylation was entirely crystalline; the yield was 3 g. It was recrystallised first from ether then from 50 % aqueous alcohol. It crystallised in white shining hexagonal platelets, m.p. 92–93°; it was very soluble in alcohol and ether, but insoluble in water; it did not reduce Fehling's solution and was neutral in reaction.

 $[\alpha]_{5461}^{25} - 30.7^\circ$ in absolute alcohol ($c = 0.717$).

Found C, 62.4; H, 8.9; OMe, 31.3 %. $C_{20}H_{34}O_7$ requires C, 62.1; H, 8.9; OMe, 32.1 %.

Simultaneous hydrolysis and methylation of trimethylbornylglucuronide methyl ester. Isolation of 2:3:4-trimethylmethyl-d-glucuronide methyl ester.

Trimethylbornylglucuronide methyl ester (6 g.) was dissolved in 100 cc. of 0.2 *N* sulphuric acid in methyl alcohol and the solution heated for 24 hours at 100° in Carius tubes. After cooling, the tubes were opened and the solution, which smelled strongly of borneol, was neutralised with silver carbonate and filtered. The methyl alcohol was removed at 40° under diminished pressure. Some crystals of borneol separated from the syrupy residue, but the bulk of it was precipitated by adding water. It was then filtered. After thorough extraction of the borneol with water, the combined aqueous extracts were concentrated *in vacuo* at 45° to a syrup. The syrup, however, still contained silver salts and these were removed by dissolving the syrup in ether and filtering the precipitated silver salts. The ethereal solution on concentrating gave a faintly yellow clear mobile syrup which was thoroughly dried and distilled (yield 3.8 g.). The syrup distilled at 131°/4 mm. giving a mobile and perfectly colourless syrup in a yield of 3.3 g. It was non-reducing and proved to be the expected 2:3:4-trimethylmethyl-*d*-glucuronide methyl ester: $n_D^{17} 1.4469$, $n_D^{15} 1.4480$; $[\alpha]_{5461}^{15} + 98.9^\circ$ in water ($c = 0.531$). Found C, 49.7; H, 7.7; OMe, 57.6 %. $C_{11}H_{20}O_7$ requires C, 50.0; H, 7.6; OMe, 58.7 %.

The borneol which separated was dissolved in ether and the solution filtered and evaporated to dryness. The residue was then purified by distillation in steam and dried in a desiccator. It melted at 206° and had $[\alpha]_{5461}^{16} + 30.1^\circ$ in absolute alcohol ($c = 1.164$). The rotation of the original borneol fed to the dogs was $[\alpha]_{5461} + 20^\circ$ in absolute alcohol; m.p. 207°.

*Oxidation of 2:3:4-trimethylmethylglucuronide methyl ester with nitric acid.
Esterification of the products of oxidation.*

The ester (2 g.) was dissolved in 20 cc. of nitric acid (sp. gr. 1.42). The reaction was initiated by heating on a water-bath to 65°. Very vigorous evolution of oxides of nitrogen took place and the mixture had to be cooled to moderate the reaction. After half an hour the temperature was raised to 90° and this temperature was maintained for 2 hours. At the end of this period the evolution of gas had ceased and the solution was diluted with an equal volume of water. The nitric acid was removed by distillation with water at 40° under diminished pressure, using a continuous feed arrangement to supply fresh quantities of water without interrupting the distillation. Finally, the solution was concentrated to a pale yellow stiff syrup with a strong acid reaction, due in part to the presence of residual traces of nitric acid. This syrup was taken up in methyl alcohol and the solvent evaporated to remove traces of water with it. The syrup was then thoroughly dried *in vacuo* over phosphorus pentoxide at 70–80°. The residual nitric acid did not cause any decomposition during drying, nor did it interfere with the subsequent esterification.

Esterification of the acid syrup was accomplished by boiling for 7 hours with 30 cc. of 4 % hydrogen chloride in methyl alcohol. The solution was next neutralised with silver oxide and dried by standing overnight over anhydrous sodium sulphate. After filtering and extracting the residues with methyl alcohol the solution was concentrated to a pale yellow mobile syrup. It still contained silver salts, derived from the residual nitric acid, and these were removed by dissolution in ether and filtering. The ethereal solution was now concentrated and the resulting syrup thoroughly dried *in vacuo*. The yield was 1.5 g.

High vacuum distillation of the esterified syrup. The dried syrup was distilled in a high vacuum and three fractions collected as follows:

Fraction	Bath temperature	Pressure mm.	Yield g.	Remarks
1	115–119°	0.24	0.531	Colourless mobile syrup
2	120–136°	0.25	0.358	Ditto
3	140–150°	0.34	0.497	Viscous yellow syrup

A small amount of a dark residue remained in the distilling flask and was neglected. Each fraction was then examined separately.

Examination of fraction 1.

The refractive index was n_D^{14} 1.4409; $[\alpha]_{5461} + 18.72^\circ$ in methyl alcohol ($c = 1.602$). Found OMe, 61.56 %. These values accord with those required for a mixture consisting of 80 % of dimethyl *i*-xylotrimethoxyglutarate [Hirst and Purves, 1923] and 20 % of dimethyl *d*-dimethoxysuccinate [Haworth, Hirst and Miller, 1927] (the former has n_D^{15} 1.4402 and $[\alpha]_D 0^\circ$, and the latter has n_D^{20} 1.4340 and $[\alpha]_D 81^\circ$ in methyl alcohol). A mixture of these two esters in the above mentioned proportions gives n_D^{14} 1.4398, $[\alpha]_D + 16.2^\circ$ and OMe, 61.64 %.

Action of ammonia on fraction 1. 0.4 g. of the syrup was dissolved in 10 cc. of dry methyl alcohol, and the solution was saturated with ammonia at 0°. The solution developed a light pink colour and deposited crystals in 24 hours; these were filtered, washed thoroughly with methyl alcohol followed by ether and dried. They formed tufts of needles and were identified as *d*-dimethoxy-succindiamide [Haworth, Hirst and Miller, 1927]. The solution was then re-treated with dry ammonia, and a second crop of crystals, differing in appearance from the first, was collected over a period of 14 days. The solution had also

developed, in one experiment, a deep purplish colour, typical to the formation of the xylotrimethoxyglutardiamide [see Hirst and Purves, 1923]. On evaporating the solution to dryness a further amount of the second crystalline material was collected. Some difficulty was experienced in purifying the last crop of crystals, but this was eventually accomplished by dissolving in ethyl alcohol, decolorising with charcoal and then filtering the solution. The solution was then evaporated to dryness and the resulting sticky crystals washed with cold methyl alcohol followed by ether, to remove adhering syrup. These crystals were identified as *i*-xylotrimethoxyglutardiamide [Hirst and Purves, 1923].

Analysis, etc.

1. *d*-Dimethoxysuccindiamide. Yield 0.08 g.; M.P. 272°; $[\alpha]_{D}^{16} + 114.6^{\circ}$ in water ($c = 0.193$); $[\alpha]_D = +97.1^{\circ}$ (calc.). $[\alpha]_{D}^{19} + 113.2^{\circ}$ in water ($c = 0.159$); $[\alpha]_D 95.9^{\circ}$ (calc.). Found OMe, 35.8; N, 15.9 %. $C_6H_{12}O_4N_2$ requires OMe, 35.2; N, 15.9 %.

2. *i*-Xylotrimethoxyglutardiamide. Yield 0.1 g.; M.P. 195° to a blue melt. It was optically inactive. Found OMe, 42.1; N, 12.97 %. $C_8H_{16}O_5N_2$ requires OMe, 42.3; N, 12.73 %.

Examination of fraction 2.

The refractive index was $n_D^{14} 1.4430$; $[\alpha]_{D}^{17} 18.37^{\circ}$ in methyl alcohol ($c = 1.143$). Found OMe, 59.26 %. These values show it to possess almost the same composition as fraction 1.

Action of ammonia on fraction 2. 0.25 g. of the syrup was treated in the same way as fraction 1. The solution turned a pale greenish-blue colour indicating the presence of the xylo-derivative. A small amount of dimethoxysuccindiamide was isolated followed by the xylotrimethoxyglutardiamide and was examined as in fraction 1.

Examination of fraction 3.

Isolation of 2:3:4-trimethyl-8-saccharolactone methyl ester. The refractive index of the syrup was $n_D^{14} 1.4570$ and the methoxyl content OMe, 55.63 %. The methoxyl value suggested that it was, most probably, a mixture of trimethyl-saccharolactone methyl ester and dimethyl *i*-xylotrimethoxyglutarate. After standing for 4 days in a desiccator the syrup became partially crystalline. It was then treated with ether in small quantities, and the adhering syrup was removed, leaving a mass of white, shining platelets sparingly soluble in ether. These were thoroughly washed with ice-cold ether, dried in a desiccator and identified as 2:3:4-trimethyl-8-saccharolactone methyl ester [cf. Robertson and Waters, 1931; Charlton *et al.*, 1931]. The crystals melted at 106.4° and had $[\alpha]_{D}^{15} + 175.9^{\circ}$ in benzene ($c = 0.216$).

Titration of total carboxyl. 9.55 mg. of the lactone were boiled for half an hour with 10 cc. *N*/70 NaOH and then the solution was titrated with *N*/70 sulphuric acid. Found 5.23 cc. of *N*/70 NaOH; $C_{10}H_{16}O_7$ requires 5.39 cc. Found C, 48.4; H, 6.6; OMe, 50.6 %. $C_{10}H_{16}O_7$ requires C, 48.4; H, 6.5; OMe, 50.0 %.

Robertson and Waters [1931] quote M.P. 106° and $[\alpha]_{D}^{25} + 176.05^{\circ}$ in benzene, while Charlton *et al.* [1931] quote M.P. 107° and $[\alpha]_D^{18} + 146.5^{\circ}$ in benzene.

SUMMARY.

β -Bornyl-*d*-glucuronide (borneolglucuronic acid) $C_{16}H_{26}O_7 \cdot 1.5H_2O$, isolated as the zinc salt from the urine of human beings and dogs fed with borneol, gives crystalline 2:3:4-trimethyl- β -bornyl-*d*-glucuronide methyl ester by methylation with silver oxide and methyl iodide. This ester is converted into a mixture

of α - and β -2:3:4-trimethylmethyl-*d*-glucuronide methyl esters by the action of 0.2*N* sulphuric acid in methyl alcohol at 100° under pressure. Oxidation of the fully methylated glucuronic acid with nitric acid yields *d*-dimethoxysuccinic acid, *i*-xylotrimethoxyglutaric acid and 2:3:4-trimethyl- δ -saccharolactone. The first two of these were identified as the crystalline diamides, while the saccharolactone was identified as the crystalline methyl ester. The isolation of *i*-xylotrimethoxyglutaric acid and 2:3:4-trimethyl- δ -saccharolactone establishes a pyranoid structure for the glucuronic acid residue of bornylglucuronide, a typical conjugated glucuronic acid, synthesised in the animal body.

The expenses of this work were in part defrayed by a grant from the Medical Research Council. One of us (R. T. W.) is indebted to the Council for a whole-time assistance grant.

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CLXII. THE BIOCHEMISTRY AND PHYSIOLOGY OF GLUCURONIC ACID.

II. THE METHYLATION OF GLUCURONE OF ANIMAL ORIGIN.

By JOHN PRYDE AND RICHARD TECWYN WILLIAMS.

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(Received July 7th, 1933.)

d-GLUCURONE is the stable anhydride of *d*-glucuronic acid and it is in the form of this lactone that glucuronic acid is generally obtained. It was first prepared by Schmiedeberg and Meyer [1879] from camphoglycuronic acid isolated from the urine of dogs fed with camphor. Free glucuronic acid was not isolated in a crystalline condition until 1925 [Ehrlich and Rehorst, 1925]. *d*-Glucurone has been isolated from both plant and animal sources, and it is clear that the products from both sources are identical. *d*-Glucurone probably possesses both a pyranoid and a furanoid ring, since most of its properties indicate it to be a γ -lactone. However, direct chemical evidence on this point is lacking.

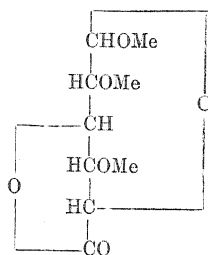
The preparation of d-glucurone. Quick [1927] has hydrolysed, with dilute sulphuric acid, bornylglucuronide obtained from the urine of dogs receiving borneol. He obtained a product which by titration he found to be a mixture of 70 % of glucuronic acid and 30 % of glucurone. Adopting Quick's method in the present investigation identical results have been obtained. The lactone is separated from this mixture by crystallisation from glacial acetic acid, and the crystals so obtained are recrystallised from water.

In the work here described it was necessary to obtain the lactone in good yield and free from the acid. Quick's method was inconvenient and necessitated the use of an acid solvent—acetic acid—which tended to char the product. A method was therefore devised by means of which the glucuronic acid residue of bornylglucuronide could be obtained entirely as the lactone (see experimental section). The glucurone obtained in this way possessed the physical properties previously quoted for this compound.

The methylation of d-glucurone. While this investigation was in progress, the methylation of *d*-glucurone, obtained by the hydrolysis of gum arabic, was described by Challinor *et al.* [1931]. These workers used methyl sulphate and alkali as methylating reagents and obtained trimethyl- β -methyl-*d*-glucuronide, a result which indicated that the lactone ring had been opened, as is to be expected when these methylating reagents are used. In the present investigation *d*-glucurone was methylated with silver oxide and methyl iodide. The products of methylation consisted of two crystalline solids, which at first were erroneously assumed to be stereoisomeric trimethylglucuronides [Pryde and Williams, 1931; 1933, 1], and an uncrystallisable syrup. Out of a total of seven methylation experiments, crystalline trimethylglucurone was isolated in three instances, while the other four experiments yielded another crystalline solid which has been provisionally named trimethylglucuralone. In each methylation the solid

product was obtained in a yield of 25 % of the glucurone used, whilst the other 75 % underwent more extensive methylation, as a result of the opening of the lactone ring. In no case were trimethylglucurone and trimethylglucuralone isolated together from the same methylation. The conditions under which either was formed seemed to be identical, but it is probable that some slight variation in the conditions of the methylation which would favour the formation of one or the other of these two solids has been overlooked by us. This problem is at present the subject of further investigation.

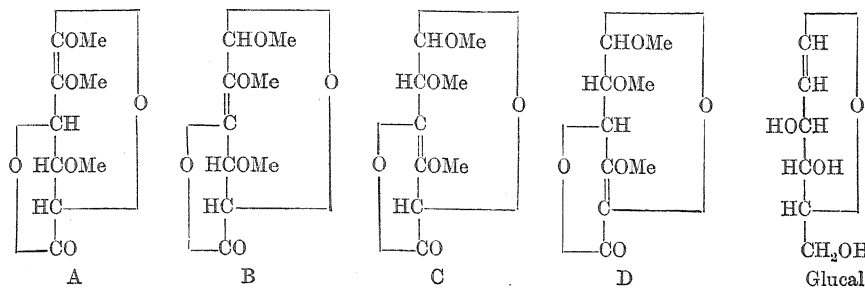
Trimethyl-d-glucurone. This compound was isolated after subjecting *d*-glucurone, dissolved in methyl alcohol, to two or three methylations, each of 8 hours' duration, with silver oxide and methyl iodide. It formed very long colourless prismatic needles of m.p. 131–132°. It was purified by recrystallisation from ether or methyl alcohol, preferably ether containing a little methyl alcohol. It was easily soluble in methyl alcohol and water but sparingly soluble in ether. It had $[\alpha]_{5461} + 197.5^\circ$ in water, which remained constant. Dissolved in methyl alcohol containing 1 % HCl, it showed a marked change in rotation at room temperature, the rotation falling according to a reaction of the first order. Its aqueous solution was neutral to litmus and Congo red papers, but was acid to methyl red indicator solution. It titrates as a lactone with alkali; it gives a positive reaction with naphthoresorcinol; it does not decolorise permanganate or bromine water like trimethylglucuralone (*vide infra*). Trimethylglucurone probably has the constitution:



There is, however, no direct chemical evidence for the existence of the furanoid ring, although it is the most probable structure. The presence of a pyranoid ring may be inferred from the results already described in Part I of this series [Pryde and Williams, 1933, 2] and from those of Challinor, Haworth and Hirst [1931]. The writers hope to supply, in the near future, definite chemical evidence on this point.

Trimethylglucuralone. This compound was isolated as a crystalline solid from the methylation of *d*-glucurone with silver oxide and methyl iodide as has already been mentioned. It was crystallised from ether or methyl alcohol and formed elongated flat tablets, m.p. 88°, soluble in methyl alcohol and dissolving with initial difficulty in water; it is sparingly soluble in ether. Its rotation in water was $[\alpha]_{5461} + 110.8^\circ$, which remained constant. Unlike trimethylglucurone it does not undergo any change in rotation in methyl alcohol containing 1 % HCl, even on heating to 100° in a sealed tube. It gives a positive naphthoresorcinol reaction and titrates as a lactone with alkali. The facts that its aqueous solution decolorises permanganate immediately and bromine water a little more gradually were taken to indicate the presence of a double bond. Several analyses for C and H were carried out and although the analytical figures did not agree with those calculated for trimethylglucurone, $C_9H_{14}O_6$, they were consistent between themselves and indicated a compound with two hydrogen atoms less, $C_9H_{12}O_6$. This

formula can be accommodated by a trimethylglucurone structure *minus* two hydrogen atoms. The following structure, A, is tentatively advanced, and it is suggested that this compound be called trimethylglucuralone on analogy with the glucals. A double bond is introduced to account for its action on permanganate and bromine water. This suggestion is supported by the fact that the introduction of an unsaturated linkage into a compound causes a marked change in optical rotation [*cf.* Stewart, 1919] and properties (*e.g.* rotation, trimethylglucurone + 197.5°; trimethylglucuralone + 110.8°; M.P. 132 and 88° respectively). This double bond could be in positions 1:2, 2:3, 3:4, 4:5 as shown by the structures A, B, C and D.



If the compound has a double bond in the position indicated by the structure B, C or D, then, since carbon atom 1 in each of these structures possesses the mutarotating groups OMe and H, mutarotation might be expected on heating the compound with acid methyl alcohol. Reference to structure A will show the H atom of the first carbon atom to be absent, making mutarotation in this case impossible. Under the conditions already specified trimethylglucuralone shows no mutarotation, and therefore of the four structures A appears most probable and is tentatively adopted here. The introduction of a 1:2 double bond into a sugar derivative in the course of methylation is ascribed to the mild oxidising action of silver oxide.

The residual syrup. The main product of the methylation of glucurone was a yellow syrup which, after separation of the crystalline material, possessed a methoxyl value of 46–47 %. This syrup on further methylation gave a product of methoxyl value approaching the theoretical value (58.7 %) for a fully methylated glucuronic acid. Three specimens of the further methylated syrup, irrespective of whether trimethylglucurone or trimethylglucuralone had been isolated, showed rotations of $[\alpha]_{5461} + 30^\circ$ in water and had refractive indices, $n_D^{18} 1.4461$, $n_D^{18} 1.4465$ and $n_D^{16} 1.4679$. The first two values for the refractive index agree with that for the α - and β -mixture of trimethylmethylglucuronide methyl ester obtained by the action of sulphuric acid in methyl alcohol on trimethylbornylglucuronide methyl ester [Pryde and Williams, 1933, 2], namely $n_D^{17} 1.4469$. This syrup was probably predominantly the β -isomeride of trimethylmethylglucuronide methyl ester, but it was not further investigated.

EXPERIMENTAL.

The preparation of d-glucurone. Bornyl glucuronide, isolated from the urine of dogs fed with borneol, was hydrolysed according to the method of Quick [1927]. The resulting mixture of glucuronic acid and its lactone was dissolved in glacial acetic acid (Quick). The crystals which separated on standing were brown in

colour and were recrystallised from water, decolorising with charcoal; M.P. 177–178°; $[\alpha]_{5461}^{17} + 20.7^\circ$ in water ($c = 0.59$).

Preparation of glucurone directly from bornylglucuronide and free from glucuronic acid. Quick's method does not give good yields when glucurone free from glucuronic acid is required. To attain this, 30 g. of bornylglucuronide were hydrolysed with 600 cc. of 0.2N H_2SO_4 by boiling for 4 hours. The solution was set aside to cool and the borneol recovered by filtration. The filtrate was treated with sufficient baryta solution to remove the H_2SO_4 quantitatively. The exact removal of H_2SO_4 is important since small residual quantities of this acid lower the yield of glucurone considerably. After standing for a short time the solution was filtered and concentrated *in vacuo* at 40° to a syrup. The syrup was taken up in the smallest quantity of water possible and sufficient alcohol was added to induce an incipient precipitation. The crystals formed on keeping in a refrigerator were filtered off and dried. The mother-liquors were put through the same sequence of operations and a further crop of glucurone obtained. The yield was 9–10 g.; M.P. 177–178°; $[\alpha]_{5461}^{17} + 21.3^\circ$ in water ($c = 1.33$); $[\alpha]_D$ calc. + 18°. In this way pure specimens of glucurone can be obtained without further recrystallisation.

The methylation of d-glucurone. Isolation of trimethylglucurone and trimethylglucuralone. A typical methylation was carried out as follows. Glucurone was methylated with excess MeI and Ag_2O , using MeOH as an extraneous solvent in the initial stages of the methylation. Each methylation was carried out for 8 hours and extraction of the products was performed with methyl alcohol and finally with dry ether. After 3 (sometimes 2) methylations, crystals were isolated in a yield of 25 % of the glucurone used. The residue after concentration of the extracts consisted of a mass of crystals embedded in a syrupy matrix. By shaking this syrupy mass with cold ether, the syrup dissolved leaving the crystalline material. The latter was recrystallised from ether containing a little methyl alcohol and dried in a desiccator.

In three cases out of seven the crystals were trimethylglucurone, M.P. 131–132° (needles); $[\alpha]_{5461}^{16} + 197.5^\circ$ in water ($c = 0.76$). Found C, 49.6; H, 6.5; OMe, 42.4 %; $C_9H_{14}O_6$ requires C, 49.5; H, 6.5; OMe, 42.7 %. 15.28 mg. of the lactone required 4.75 cc. of N/70 NaOH; calculated for $C_9H_{14}O_6$, 4.8 cc.

Trimethylglucurone (0.0467 g.) was dissolved in 10 cc. of methyl alcohol containing 1 % HCl and the change in rotation observed polarimetrically at constant temperature (20°). The initial rotation was $[\alpha]_{5461}^{20} + 197^\circ$ (after 5 mins.) falling to $[\alpha]_{5461}^{20} + 43.5$ in 190 mins. The fall in rotation followed the equation for reactions of the first order and therefore was due to mutarotation. The solution was then heated for 2 hours in a sealed tube at 100° to secure a constant equilibrium value, which was found to be $[\alpha]_{5461}^{20} + 10.7^\circ$.

Trimethylglucurone did not decolorise permanganate solution or bromine water.

In the other four cases the crystalline solid was trimethylglucuralone; M.P. 88°; $[\alpha]_{5461}^{18} + 110.8^\circ$ in water ($c = 0.63$). Found C, 50.2, 50.0, 49.9, 50.2, 50.15; H, 5.6, 5.8, 5.7, 5.6, 5.65; OMe, 43.4 %. $C_9H_{12}O_6$ requires C, 50.0; H, 5.6; OMe, 43.1 %. 48.7 mg. of trimethylglucuralone required 3.3 cc. of 0.0625N NaOH; $C_9H_{12}O_6$ requires 3.5 cc.

Trimethylglucuralone (0.0346 g.) was dissolved in 10 cc. of methyl alcohol containing 1 % HCl and the rotation observed. The initial rotation was $[\alpha]_{5461}^{20} + 110^\circ$ and after 18.25 hours the rotation was unchanged. The solution was then heated in a sealed tube for 4 hours at 100° and the rotation still remained practically unchanged, being $[\alpha]_{5461}^{20} + 107^\circ$. Trimethylglucuralone in

aqueous solution decolorised permanganate solution immediately, while bromine water was decolorised gradually.

The residual syrup. On extracting with ether the mass of crystals and syrup obtained from the methylation of glucurone and evaporating the extract to dryness a yellow neutral syrup was obtained in a yield of 75 % of the glucurone methylated (found, average for six specimens, OMe, 46-47 %). These syrups were then subjected to further methylation with silver oxide and methyl iodide. After three further methylations, the syrups were recovered by ether extraction. After evaporating the solvent the resulting syrupy product was distilled in a high vacuum (found, on three specimens, OMe, 53.4, 54.7, 57.35 %; calculated for a fully methylated glucuronic acid $C_{11}H_{20}O_7$, OMe, 58.7 %); rotations $[\alpha]_{5461}^{20^\circ} + 29.6^\circ$, $+ 30.45^\circ$, $+ 30.5^\circ$ in 20 % aqueous alcohol; refractive indices, $n_D^{18^\circ}$ 1.4465, $n_D^{18^\circ}$ 1.4461, and $n_D^{16^\circ}$ 1.4679. The data given for this syrup are quoted with reserve since it has not been prepared in a satisfactorily pure state; it is probably 2:3:4-trimethyl- β -methyl-*d*-glucuronide methyl ester, $C_{11}H_{20}O_7$.

SUMMARY.

A method is given whereby glucurone free from glucuronic acid can be prepared directly by hydrolysis of bornylglucuronide obtained from the urine of dogs fed on borneol. Methylation of glucurone with silver oxide and methyl iodide gives two crystalline solids, trimethylglucurone and an unsaturated derivative, trimethylglucuralone. Structures for both these compounds are tentatively suggested. The main bulk of the glucurone undergoes more extensive methylation owing to the opening of the lactone ring.

The expenses of this research were in part defrayed by the Medical Research Council and one of us (R. T. W.) is indebted to the Council for a whole-time assistance grant.

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CLXIII. THE BIOCHEMISTRY AND PHYSIOLOGY OF GLUCURONIC ACID.

III. THE STRUCTURE OF BENZOYLGLUCURONIC ACID.

BY JOHN PRYDE AND RICHARD TECWYN WILLIAMS.

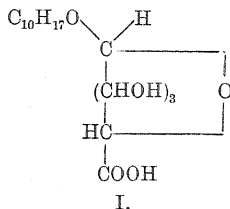
From the Physiology Institute, Newport Road, Cardiff.

(Received July 7th, 1933.)

MUCH attention has been paid to the detoxication of aromatic acids of the benzoic acid type in the animal body, but all the earlier workers failed to recognise the part played by glucuronic acid in this process, and attention was centred on the problem of the conjugation of these acids with glycine and their subsequent excretion as hippuric acids. There is now no doubt that glucuronic acid plays a very important rôle in the detoxication of aromatic acids, especially when, after the administration of large doses of the toxic acid, the preformed glycine of the body has become exhausted. The detoxication of compounds of the benzoic acid type has been the subject of detailed study by Quick [1926, 1; 1928, 1, 2; 1931; 1932, 1, 2, 3, 4].

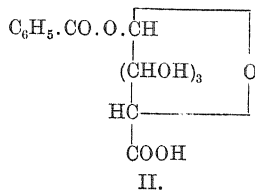
The various conjugated glucuronic acids may be divided into three types.

(1) Conjugated glucuronic acids of the glycoside-"ether" type, such as bornylglucuronide, I [cf. Pryde and Williams, 1933], in which the non-sugar residue is attached to the reducing group of glucuronic acid by means of a true glycoside link:



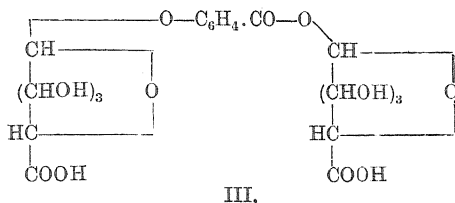
Glucuronides of this type are non-reducing.

(2) Conjugated glucuronic acids of the glycoside-"ester" type, such as benzoylglucuronide, II (benzoylglucuronic acid), in which the benzoyl radical is attached to the reducing carbon of glucuronic acid. This linkage is unstable to hydrolytic reagents and these glucuronides are reducing.

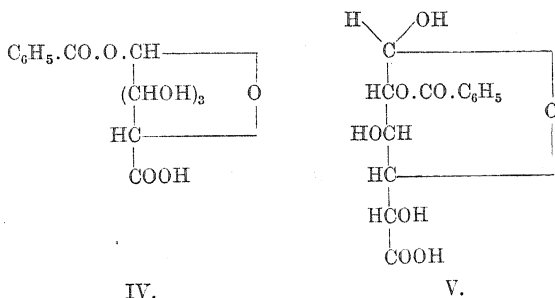


(3) Conjugated glucuronic acids containing two glucuronic acid molecules, one attached to the non-sugar residue by a glycoside-"ether" linkage as in

type (1) and the other attached by an "ester" link as in type (2); the *p*-hydroxybenzoic acid-diglucuronic acid, III, described by Quick [1932, 3] is an example of this type:



Benzoylglucuronic acid was first isolated as the sodium and strychnine salts by Magnus-Levy [1907], and he assigned to it the constitution of a 1-benzoylglucuronide, IV. The free acid was, however, prepared for the first time by Quick [1926, 2] from the urine of dogs fed with benzoic acid, but no analytical data other than titrations were given. Owing to the fact that this acid is very unstable to hydrolytic reagents, especially alkali, it reduces Fehling's solution and other alkaline sugar reagents. Quick, therefore, supposed it to be a 2-benzoylglucuronic acid, V, which would possess a free reducing (potential) aldehyde group. Various considerations led us to doubt the correctness of this view and to support the original structure suggested by Magnus-Levy (using the new amylene-oxidic ring instead of the original butylene-oxidic ring).



In the first place, on scrutinising the published data concerning glycosides containing a 1-benzoyl radical, it is found that all these glycoside-"esters" reduce Fehling's solution, owing to the ease of hydrolysis of the benzoyl group in the presence of alkali. The data are tabulated below (Table I).

Table I. *Glycosides containing a 1-benzoyl radical, which reduce Fehling's solution.*

Compound	Authority
1-Benzoyl-4 : 6-benzylideneglucose	Zervas [1931]*
1 : 2 : 3-Tribenzoyl-4 : 6-benzylideneglucose	Brigl and Grüner [1932]
1 : 2 : 3-Tribenzoylglucose	"
1 : 2 : 3-Tribenzoyldiacetylglucose	"
1 : 2 : 3 : 4-Tetrabenzoylglucose	"
1 : 2 : 3 : 4-Tetrabenzoylmonoacetylglucose	"
1 : 3 : 4 : 5-Tetrabenzoylfructose	Brigl and Schinle [1933]
1 : 3 : 4 : 5 : 6-Pentabenzoylfructose	"

* The reducing properties of this compound were not mentioned by Zervas but it was prepared by us by Zervas's method and found to be reducing.

It is also found that certain nitrogenous glycosides, such as the amino-acid- and the amino-acid ester-glycosides [Maurer and Schiedt, 1932] and glucosidureides [Haring and Johnson, 1933] reduce Fehling's solution owing to hydrolysis in the presence of alkali.

Secondly, an examination of the optical rotations of β -*d*-glucosides and the corresponding β -*d*-glucuronides shows these to be roughly of the same order. This observation accords with the expectation that the change of $-\text{CH}_2\text{OH}$ to $-\text{COOH}$ should have but a small influence on the rotation. Table II gives all the data available on this point and it will be noted that benzoylglucuronic acid and 1-benzoyl- β -*d*-glucoside [Zervas, 1931] fall in line with the others, thus giving further evidence for the 1-benzoyl-structure of benzoylglucuronic acid.

Table II. *Rotations and melting-points of d-glucosides and corresponding d-glucuronides.*

Compound	<i>d</i> -Glucoside				<i>d</i> -Glucuronide		
	$[\alpha]_D$	Solvent	M.P.		$[\alpha]_D$	Solvent	M.P.
(1) β -Benzoyl	-26.8°	Water	193°	(8)	-25.3°	Water	183°
(2) β -Phenyl	-71.7	Water	176	(9)	-82	Water	151
(3) β - <i>d</i> -Bornyl	-42.4	Alcohol	135	(10)	-37*	Water	174
(4) β - <i>l</i> -Bornyl	-60.1	Alcohol	—	(11)	-66.6	Water	—
(5) β - <i>l</i> -Menthyl	-93.6	Alcohol	76	(12)	-104.6	Alcohol	110
(6) α - <i>l</i> -Menthyl	+64	Alcohol	160	(13)	+52	Alcohol	130
(7) β -Phloroglucinyll	-74.8	Water	239	(14)	-80.8†	Water	—
(7) Urea	-23.4	Water	207	(15)	-21.2‡	Water	—

* Rotation of sodium salt.

† Rotation of potassium salt.

‡ Calculated from rotation of barium salt.

(1) [Zervas, 1931]. (2) [Fischer and Mechel, 1916]. (3) [Fischer and Raske, 1909]. (4) [Hämäläinen, 1913]. (5) [Fischer and Bergmann, 1917]. (6) [Fischer and Strauss, 1912]. (7) [Hynd, 1926; Fischer, 1914]. (8) This investigation. (9) [Salkowski and Neuberg, 1906]. (10) [Magnus-Levy, 1906]. (11) [Pryde and Williams, unpublished results]. (12) [H. Fischer, 1911]. (13) [Bergmann and Wolff, 1923]. (14) [Sera, 1914]. (15) [Neuberg and Niemann, 1905].

Quick [1926, 2] studied polarimetrically the action of NaCN on benzoylglucuronic acid (*cf.* Fig. 1) and considered that the observed changes were due

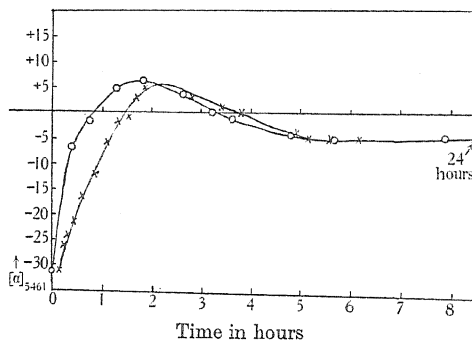
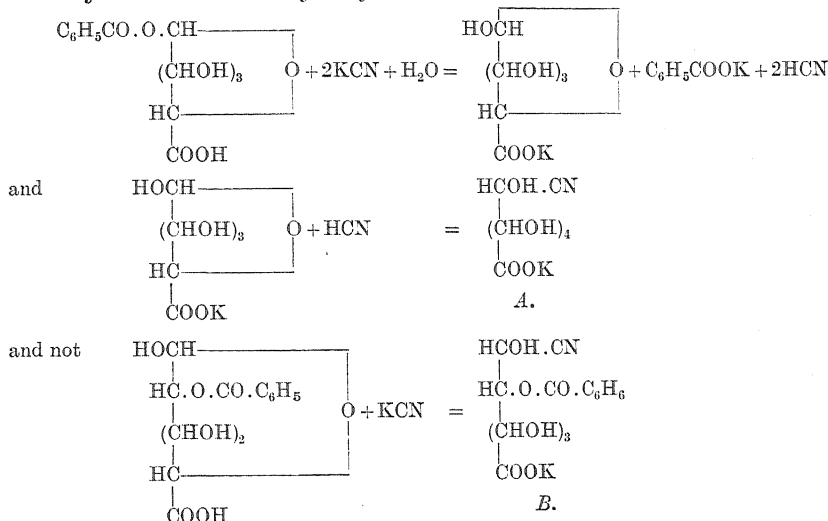


Fig. 1. The action of KCN and NaCN on benzoylglucuronic acid. ○ Quick [1926, 2] for NaCN; × Present investigation with KCN.

to mutarotation (*i.e.* of a 2-benzoylglucuronide) and the formation of a cyanohydrin of benzoylglucuronic acid, *B* (p. 1213). We have obtained similar results using KCN, but owing to the instability of the benzoyl group in the presence of

cyanide, a quite different interpretation is necessary. The alkalinity of the cyanide causes hydrolytic fission of the benzoyl radical and mutarotation and formation of the cyanohydrin of glucuronic acid, *A* (not benzoylglucuronic acid) follows. This view is supported by the fact that free benzoic acid was isolated when a solution of benzoylglucuronic acid and KCN was allowed to stand for 24 hours, and benzoylglucuronic acid does not react with HCN without splitting off benzoic acid. Since more than one molecule of cyanide [*cf.* Quick, 1926, 2] is required to react completely with one molecule of benzoylglucuronic acid, it is evident that some cyanide is used in hydrolysis.



Attempts to methylate benzoylglucuronic acid with Ag_2O and MeI, or with diazomethane failed, since the compound was hydrolysed during methylation and the benzoyl group was split off and formed methyl benzoate.

Improvements were effected in Quick's method [1926, 2] of preparing benzoylglucuronic acid, and a purer specimen of the acid was obtained, M.P. 183° (Quick quotes M.P. $170\text{--}172^\circ$).

EXPERIMENTAL.

The preparation and purification of benzoylglucuronic acid.

Benzoylglucuronic acid was prepared by Magnus-Levy [1907] as the strychnine and sodium salts from the urine of a sheep fed on sodium benzoate, while Quick [1926, 2] prepared the free acid from the urine of dogs similarly fed. In the present work 5 g. of benzoic acid were fed daily to each of several small dogs, the acid being contained in small gelatin capsules which were given with meat. The urines were collected every 24 hours and preserved with toluene. The urines were acid in reaction (p_{H} 3-4) and were laevorotatory. They were worked up every 3 days as follows. All reagents were kept cold in the refrigerator. The urine, after addition of a little acetic acid, was precipitated exactly with normal lead acetate and filtered. The filtrate was brought to p_{H} 6-7 with cold dilute ammonia and then precipitated with ice-cold saturated basic lead acetate. The precipitate was filtered immediately on a large Büchner funnel under suction and washed several times with ice-water. The precipitate was made into a thin paste with ice-water and decomposed with H_2S , and the lead sulphide filtered off through Seitz "Brilliant" asbestos. The filtrate was again treated with H_2S

to ensure complete removal of lead, which interferes with the successful crystallisation of benzoylglucuronic acid. The clear filtrate was aerated to remove H_2S and concentrated *in vacuo* until crystallisation of hippuric acid set in. The solution was then allowed to stand and the hippuric acid filtered off. At this point the filtrate was shaken with an equal volume of chloroform. The chloroform layer became red owing to extraction of pigments and also dissolved any free benzoic acid (which may be separated on evaporation of the chloroform). After separation of the chloroform layer, the aqueous layer generally deposited crystals immediately, and the crystallisation was completed in the refrigerator. The crude benzoylglucuronic acid was filtered off and recrystallised from a little hot water (decolourising with norite charcoal) and dried in a desiccator over CaCl_2 . The dried acid still contained hippuric acid and possessed a slight yellow colour. It was now pulverised (5 g.) and shaken up with ether (200 cc.). The ether was renewed three times in 24 hours; each time the acid was shaken with ether and allowed to stand until the ether was renewed. The product still contained traces of hippuric acid and was dissolved in a little methyl alcohol and the solution decolorised with norite. The filtered methyl alcohol solution was then precipitated with 20 times its volume of dry ether and the whole allowed to stand in the refrigerator to complete the crystallisation of the precipitated benzoylglucuronic acid, which crystallised gradually but completely from the solution in aggregates of short, stout, radiating needles, colourless and free from traces of hippuric acid which was retained in the ether. The ether was decanted and the crystals dried.

They had M.P. 183° ; $[\alpha]_{\text{D}}^{21} - 29.9^\circ$ in water ($c = 1.639$); $[\alpha]_{\text{D}} - 25.3^\circ$; yield 5 g. from 36 g. benzoic acid fed. The acid is soluble in methyl and ethyl alcohols and water, slightly soluble in ethyl acetate and insoluble in ether and chloroform. Found, C, 52.2; H, 4.85 %; $\text{C}_{13}\text{H}_{14}\text{O}_8$ requires C, 52.3; H, 4.7 %.

The action of KCN on benzoylglucuronic acid.

0.25 g. of benzoylglucuronic acid was dissolved in an aqueous solution of potassium cyanide. The solution was clear initially, but after about 1 min. became opalescent and then cleared up again. The whole was allowed to stand with an equal volume of chloroform in a corked flask for 24 hours at room temperature. A similar solution was made up omitting the cyanide. The chloroform layer was then separated and evaporated at low temperature *in vacuo*. A crystalline residue was obtained in the case where KCN was added but no residue was obtained in the other case. The crystalline residue consisted of typical platelets of benzoic acid (M.P. 117° on recrystallisation from ether) and gave all the qualitative tests for this acid. It is probable that the greater proportion of the benzoic acid was converted into potassium benzoate. Similar experiments were made using NaHCO_3 instead of KCN but no free benzoic acid was obtained, since it was probably converted entirely into sodium benzoate.

The action of KCN was also followed polarimetrically; 0.1312 g. of benzoylglucuronic acid was dissolved in 10 cc. of water, and 7 cc. of the solution were mixed with 2 cc. of a solution of KCN (0.1405 g. in 10 cc.) and the change in rotation observed polarimetrically. The results are given in Fig. 1, which also gives the data of Quick [1926, 2] for the action of NaCN.

SUMMARY.

Conjugated glucuronic acids can be divided into three types; the glycoside-“ether” type, the glycoside-“ester” type and a third type which is a combination of the first and second types. Benzoylglucuronic acid (a glycoside-“ester”

derivative) has been prepared in a state of purity, and full analytical data are recorded for the first time. Like all glycosides containing a 1-benzoyl radical, benzoylglucuronic acid reduces sugar reagents owing to the ease of hydrolysis of the benzoyl radical. A study of the action of KCN on benzoylglucuronic acid, and certain other theoretical considerations, suggest that it is a 1-benzoyl derivative of glucuronic acid, and not a 2-benzoyl derivative. Attempts to methylate it with methyl iodide and silver oxide and with diazomethane failed since the benzoyl group is very unstable and is split off as methyl benzoate.

The expenses of this research were in part defrayed by the Medical Research Council, and one of us (R. T. W.) is indebted to the Council for a whole-time assistance grant.

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CLXIV. A NOTE ON THE TECHNIQUE OF ASSAYING POSTERIOR PITUITARY EXTRACTS FOR OXYTOMIC ACTIVITY.

By JOHN MASSON GULLAND.

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(Received June 20th, 1933.)

THE assay of posterior pituitary extracts for oxytomic activity by the guinea-pig uterus method necessitates the use of carefully segregated, young, virgin guinea-pigs, of which the weights lie between relatively close limits. It is well known that in spite of every care and precaution the uterine horns of some apparently suitable guinea-pigs prove unsatisfactory for making the assay. From these and other considerations, it is desirable to record any procedure which reduces the wastage of potentially suitable animals.

It is not generally realised that uterine horns may be preserved in Ringer solution. From the experience of the many oxytomic assays which have been made in this laboratory during the past 18 months it is now the practice to remove both horns from every guinea-pig and to place them separately in plugged, sterile test-tubes containing Ringer solution (5-10 cc.) of the same composition as is used in the testing-bath (for composition, see Burn [1928]). The tubes are then placed in the cold store at 0-1° until the horn is to be used. It has not been customary to allow horns to remain at 0° for longer than 30 hours, although occasionally satisfactory contractions have been obtained with older preparations. During the assay horns treated as described above completely resemble those which are placed in the bath immediately after their removal from the animal.

By following this procedure both horns of every suitable guinea-pig may be utilised when only one testing bath is in use. The saving in animals is obvious.

The following modification of the standard apparatus [Burn and Dale, 1922; Burn, 1928] simplifies the assay of oxytomic activity. The outlet of the bath is joined by a sleeve of rubber tubing (see Fig. 1) to a two-way glass tap; one limb of this (*B*) can be closed by a pinch-clip and is also fitted with a side-tube (*C*) as close to the barrel of the tap as possible. Warm Ringer solution from the reservoir enters at (*C*) by a siphon which has neither tap nor pinch-clip.

The following sequence is observed for each contraction. (1) The bath is emptied through the outlet (*A*), and simultaneously the siphon from the reservoir is flushed out with warm Ringer solution by opening the pinch-clip; (2) the pinch-clip being closed, the bath is filled by turning the barrel of the glass tap through 180°, and then through 90° to close the tap.

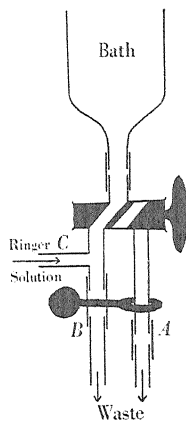


Fig. 1.

The advantages of this modification are (*a*) simplicity and speed of manipulation, and (*b*) the avoidance of a "dead" space in the tube beneath the bath; this space, if present, is not flushed out with fresh Ringer solution, and thus spent Ringer solution and pituitary extract are inevitably carried back into the bath by the fresh supply of Ringer solution from the reservoir.

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CLXV. THE OXYTOMIC HORMONE OF THE POSTERIOR LOBE OF THE PITUITARY GLAND.

II. THE ACTION OF NITROUS ACID AND NITRIC ACID.

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(Received June 27th, 1933.)

It has frequently been recorded that the addition of a base-precipitating reagent to a crude or partially purified pituitary extract removed the oxytomic hormone in association with the precipitates formed by organic bases (possibly proteoses or peptones [Abel and Pincoffs, 1917]). This fact led to the view that the hormone itself is a base, till Dudley [1923] drew attention to the unsatisfactory character of this evidence in view of the ease with which the hormone is absorbed by various precipitates.

The apparent destruction of oxytomic activity by proteolytic enzymes such as trypsin and papain, which occurs when preparations of these enzymes act on the hormone [Dudley, 1919; Dale and Dudley, 1921; Thorpe, 1926; Freudenberg *et al.*, 1932] has been regarded as indicative of a peptide structure. Polypeptides which occur naturally usually contain free amino- or basic imino-groups.

Thus, although the evidence for the presence of a basic group was indecisive, it was desirable to study the behaviour of the hormone with nitrous acid. Hitherto, apart from sodium hydroxide and hydrochloric acid, chemical reagents have not been used with the deliberate intention of destroying the oxytomic substance, and the suitability of nitrous acid for these pioneer experiments was enhanced by the ease with which it can be estimated and by the fact that it may be employed at the hydrogen ion concentration at which the hormone is most stable [Adams, 1917; Gaddum, 1930].

Preliminary experiments with a weak commercial posterior lobe extract (10 international oxytomic units per cc.) showed that the oxytomic activity was lowered to about 20 % of its initial value by treatment with nitrous acid at p_H 3 for about 30 hours. It was evident that the true significance of the interaction with nitrous acid could only be assessed by following the progressive fall in physiological activity with the passage of time. Moreover, it was necessary for the following practical reasons to use preparations of the hormone in which high oxytomic activity was associated with small amounts of organic solids in concentrated solution [Gulland and Newton, 1932]: sodium nitrite in concentrations greater than a certain value would have an effect on the isolated uterus during the assay; inactive glandular material would potentially exhaust the supply of nitrous acid unnecessarily and might necessitate the presence in the testing

bath of excessive quantities of salts (hypertonic solutions); the oxytomic value might fall to much less than 20 % of its initial value.

In Exps. 1 and 2 an initially large excess of nitrous acid was permitted to react with a hormone preparation in ice-cold acetate buffer at p_H 3. Samples were brought to p_H 7.4 after withdrawal, in order to arrest the action of nitrous acid, and were then preserved at 0° for the oxytomic assay. Samples mentioned in this paper were assayed by the degree of contraction they produced in the isolated uterus of the guinea-pig, the method followed being that of Dale and Laidlaw [1912] as modified by Burn and Dale [1922] and Burn [1928]. The method of grouping the contractions illustrated in a previous communication [Gulland and Newton, 1932] was adopted in every case, except in Exp. 3 as stated.

In the first experiment (Fig. 1) the oxytomic activity had fallen to 32 % of its initial value in the course of 3 minutes, the point at which the first sample was taken. Thereafter it slowly diminished to 16-18 % at 48 hours and remained

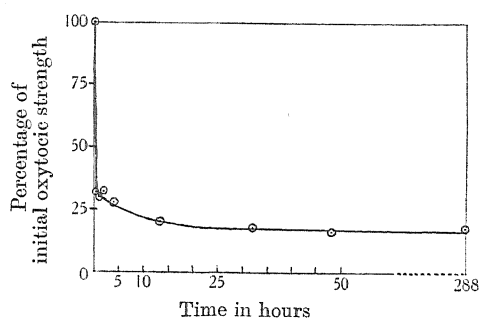


Fig. 1.

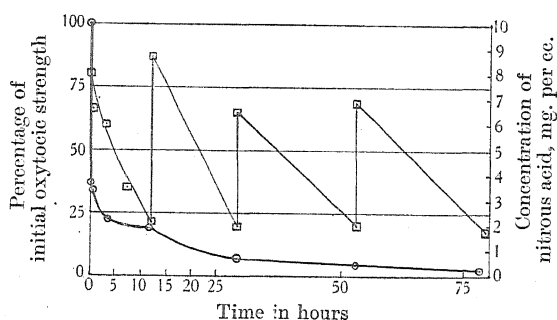


Fig. 2.

constant at that strength (Table I). An estimation of nitrous acid made at 48 hours showed that only 6 % of the original amount remained. Two explanations of these results are therefore possible; nitrous acid may have a dual effect on the hormone, or, alternatively, its action may reduce the potency to 18 %, the flattening of the curve being the combined results of progressive diminution of active substance and of reagent.

In the second experiment (Fig. 2), which was designed to distinguish between these alternatives, a large excess of nitrous acid was again used, but in this case

its concentration was maintained relatively high by the replacement with fresh nitrite solution of the volumes removed for assay. Both residual oxytotic activities and concentrations of nitrous acid were estimated throughout the reaction (Table II). In the course of 1 minute the activity fell to 37 % of its initial value and after 15 minutes was still 34 %. It is important to note that no significant diminution of the large excess of nitrous acid had occurred even after 25 minutes had elapsed from zero.

After a 3-hour period of less violent inactivation the rate of destruction then decreased markedly, and the oxytotic activity remained almost constant for 8 hours (falling from 22 to 19 % between $3\frac{1}{2}$ and $11\frac{1}{2}$ hours). During the first 12 hours of the experiment the nitrous acid diminished steadily, but at that point its concentration was still 2.1 mg. per cc., the ratio of the weights of solid associated with the hormone and of nitrous acid being 1:26. It is evident therefore that the markedly decreased inactivation amounting almost to stabilisation of physiological value, which occurred between $3\frac{1}{2}$ and $11\frac{1}{2}$ hours, had taken place in presence of a considerable excess of nitrous acid.

The addition at 12 hours of nitrite solution, sufficient to restore the concentration of nitrous acid to more than its initial value, then produced a renewed increase in the rate of oxytotic inactivation. This increased rate was not maintained, however, and during the latter half of the experiment the oxytotic activity fell slowly but steadily whilst the concentration of nitrous acid was twice restored almost to its original value. This experiment was not prolonged to include activities of less than 2 % (see, however, Exp. 3).

It will be observed that although it has not been possible to maintain the concentration of nitrous acid at a uniform value during Exp. 2, the average (about 4 mg. per cc.) is a large excess as compared with the weight of glandular solid (0.08 mg. per cc. at the beginning of the experiment), of which only a small part can possess oxytotic properties. The apparently large utilisation of nitrous acid during the experiment is undoubtedly the result of that decomposition of nitrous acid which occurs in aqueous solutions when exposed to air [Rây *et al.*, 1917; Klemenc and Pollak, 1922; Taylor *et al.*, 1927].

In a subsequent experiment (Exp. 3) in which the nitrous acid was renewed repeatedly during a week the oxytotic strength was 0.15–0.2 % after 2 days and about 0.1 % after 7 days. It is difficult to assay accurately such feebly active solutions, and almost impossible to do so when, as is the case here, salts are present in quantities sufficient to upset the isotonicity of the fluid in the testing bath. These values are therefore based on a direct comparison of the heights of contractions evoked by a few selected doses of the experimental solution and standard. It is evident that even after prolonged treatment with nitrous acid the decomposition products of the hormone still retain an oxytotic effect, which, although small as compared with that due to the hormone itself, is by no means inconsiderable. The solid present in a dose used in the assay of Exp. 3 produced a 60 % contraction at a dilution of 1 in 30,000 in the testing bath; moreover, the preparation used in this experiment contained only 45 % of the activity of the most potent preparation of Gulland and Newton [1932], which itself was undoubtedly far from being pure hormone. Clearly, therefore, the active substance resulting from the prolonged destructive action of nitrous acid could, if obtained pure, exert an oxytotic effect at a great dilution. It is possible, however, that this substance is present in the original pituitary solution and that it is not a decomposition product of the hormone.

An explanation of the results of Exps. 1, 2 and 3 may be sought by considering them from three aspects; of these, the first only is acceptable.

(1) Nitrous acid and the products of its oxidation or decomposition may have a triple action on the hormone molecule. The first attack is very rapid and produces a substance having an activity equivalent to about 35 % of that of the hormone; the second reaction is less rapid, apparently proceeds in presence of relatively small concentrations of nitrous acid as compared with the third reaction and produces a substance having an activity of about 20 % of that of the hormone; the third reaction is slow, requires a large excess of nitrous acid and reduces the activity to about 0.1 % of that of the hormone. These reactions would presumably occur concurrently. The differentiation between the second and third reactions is not quite sharp from these results, but it is also supported by the results of Exp. 4 (below), and is confirmed in Exps. 5 and 6.

(2) Nitrous acid may have one action on the hormone, reducing the oxytomic activity to a fraction (less than 1 %) of its initial value. In this case there would be two variables, *viz.* the concentrations of nitrous acid and of oxytomic substance, and since the concentration of nitrous acid remains high and relatively constant over the early part of Exp. 2, the oxytomic value should have become very small in (say) 5 minutes. In fact, however, it is still 34 % after 25 minutes have elapsed from zero, and this explanation is therefore invalid.

(3) Nitrous acid may have one action only, which reduces the oxytomic value to 20 %. The residual fall in activity, as shown for example in Fig. 2, would then be due to decomposition of the product at p_H 3 in the experimental solution irrespective of the presence of nitrous acid, or at p_H 7.4 in the samples. The p_H -stability of the oxytomic substances in samples from Exp. 1 has been tested. The samples taken at 1 hour (30 % activity) and at 48 hours (16–18 %) were both unchanged in activity after remaining at p_H 7.4 for a week. A sample taken at 48 hours was stable at p_H 3 for 12 days.

The alternative explanations involving a single action of nitrous acid are thus untenable, and it is concluded that the hormone undergoes three transformations during the action of nitrous acid. The possibility has not been overlooked that facile contraction of the uterus is only evoked by two or more hormones acting in unison and that these are destroyed at different rates by nitrous acid. This explanation seems improbable, and the simpler alternative is preferable as a basis for further investigations.

An attempt was made (Exp. 4) to study the p_H -stability of the first product (35 % activity) of the action of nitrous acid by destroying the reagent with an excess of glycine at 37°. The desired end was not attained, since the increased temperature naturally hastened the inactivation of the hormone by nitrous acid or the products of its decomposition as well as the destruction of nitrous acid by glycine; by the time that the concentration of nitrous acid had become extremely small, the oxytomic activity had decreased to 20 %. It is, however, noteworthy that this value also represents the state of activity in Exps. 1 and 2 when nitrous acid is present in small, but definite, amounts. As already mentioned, this supports the existence of three reactions between the hormone and nitrous acid or its decomposition products.

It was clear that the chief barrier to a complete differentiation of these three reactions was the variable concentration of nitrous acid in Exps. 1 and 2. An experiment was therefore carried out (Exp. 5), in which the hormone in acetate buffer at p_H 3 was subjected for 30 hours to the action of nitrous acid at concentrations which fluctuated only between 1.4 and 1.6 mg. per cc. (Fig. 3). The concentration of nitrous acid was estimated at approximately hourly intervals, the loss through decomposition being made up by the addition of sodium nitrite solution; samples were withdrawn for oxytomic assay at appropriate times. This

experiment thus represents the closest practicable realisation of a study of the inactivation of the hormone in presence of a constant concentration of nitrous acid. Here, as in the previous experiments, the hormone suffered an extremely rapid inactivation during the first few minutes, and in the course of about 40 minutes the activity had decreased to about 35 % of its initial value. Thereafter the inactivation proceeded slowly throughout the experiment, and the

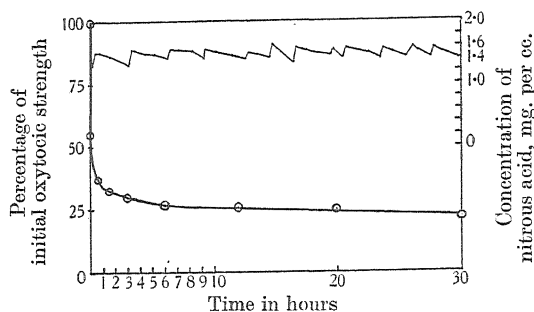


Fig. 3.

oxytocic value was still 22 % after 30 hours. This experiment, considered in conjunction with those described above, demonstrated conclusively that two reactions are needed to reduce the oxytocic value to 20 % and makes it probable that the second of these (causing a fall from 35 to 20 %) is not the same as the slow reaction (20 to 0.1 %) observed in Exp. 2.

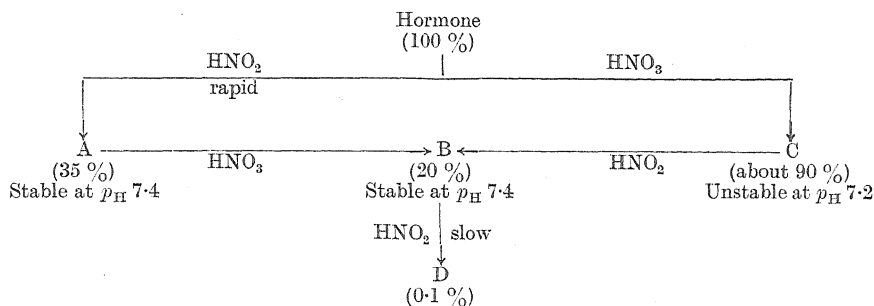
The sole factor which has not been considered is the action of the decomposition products of nitrous acid. It is generally accepted that in contact with air there is a certain amount of oxidation accompanied by the reaction $3\text{HNO}_2 \rightarrow \text{HNO}_3 + 2\text{NO} + \text{H}_2\text{O}$ [Montemartini, 1890]. The ultimate products of the oxidation of nitric oxide by air in presence of water are nitrous and nitric acids, so that the decomposition product of nitrous acid which is likely to be of importance in the present instance is nitric acid. The oxytocic activity falls to an estimated value of about 90 % when the hormone is acted on at 0° by a solution of sodium nitrate at p_{H} 3 (Exp. 6). The technique used in the nitrous acid experiments was adopted here, and the assay is complicated by the instability at p_{H} 7.2 of the product of this reaction and its consequent destruction in the samples. Two important points emerge from this experiment.

In the first place, the 42-hour sample contained more of the unstable product of the reaction than did the 22-hour sample, because its inactivation at p_{H} 7.2 was very much greater in a shorter time. Secondly, the product of the action of nitric acid is not the same as the first, rapidly formed product of the action of nitrous acid, because in Exp. 1 the sample containing the latter substance was assayed immediately and the activity remained unchanged for a week at p_{H} 7.4. This excludes the possibility that the inactivation at the beginning of Exp. 6 is due to small amounts of nitrous acid formed by the reduction of nitric acid by the hormone or by inactive glandular material.

The activity of the 88-hour sample in Exp. 6 appears to be lower than might be expected. If this is so, it may be due to p_{H} -instability of the sample or to the action of traces of nitrous acid formed by the reduction of nitric acid. For these reasons it would be difficult to ascertain the oxytocic strength of the product of the action of nitric acid, but uncertainty as to the true value does not affect the present discussion.

From the latter part of Exp. 6 it was ascertained which of the three successive inactivations of the hormone in presence of nitrous acid is due to nitric acid. At 89 hours solid sodium nitrite was added in amount equivalent to a concentration of 2.5 mg. of nitrous acid per cc. of the reaction mixture. It was estimated that this amount of nitrous acid would have been sufficient to reduce the activity of the hormone to 20–25 % if added directly to its solution. The mixture was re-adjusted to p_H 3 and preserved at 0°. 70 hours later free nitrous acid was still present, and the activity was found by assay to have fallen to 25 % of the initial value of Exp. 6. Several days later, when nitrous acid could no longer be detected, the activity had become 20 %. If nitric acid is responsible for the slow inactivation which occurred, for example, at the end of Exp. 2, then the value at the end of Exp. 6 would have been far below 20 %. If nitric acid causes the fall in activity from 35 to 20 % (Exps. 1, 2 and 5), then the predicted value for the activity at the end of Exp. 6 would have been 20 %. Clearly, therefore, the fall from 35 to 20 % is due wholly or in part to the action of nitric acid. It is not possible at present to be certain that this phase of the inactivation is solely due to nitric acid. It should be noted that the substance of 20 % activity in Exp. 6 was stable at p_H 7.2 and 0°.

The following diagram summarises the decompositions which the hormone and its derivatives undergo when nitrous acid is added to its aqueous solution at p_H 3 when exposed to air. It is considered probable that the substance B is obtained irrespective of the order in which nitrous and nitric acids react with the hormone, in view of the oxytomic value of B when prepared by either route, its stability as compared with that of C, and the unavoidable presence of nitric acid in a nitrous acid solution after exposure to air.



Valuable evidence from the constitutional standpoint may be drawn from the fact that the oxytomic activity may be destroyed in stages, or in other words, that the nature of the hormone is such that it is possible to prepare a series of derivatives which possess the power of contracting smooth muscle. This fact, which has not hitherto been demonstrated, forms the starting point for further investigations.

In view of the manifold activities of nitrous acid in acetic acid solution, for example, its interaction with amines, imines, phenols, amino-alcohols and active methylene groups, it is impossible from the available evidence alone to deduce the presence of definite structures in the hormone molecule. It will be recalled, however, that amino-groups in the α -position to carboxyl groups or peptide linkages react rapidly with nitrous acid, whereas the velocity decreases progressively as the amino-group is moved away from the carboxyl along a carbon chain; thus, Dunn and Schmidt [1922] find that at 5° the α -amino-group of alanine reacts about three times more rapidly than does the ϵ -amino-group of

lysine, which in turn surpasses in reactivity the guanidine group of arginine [Plimmer, 1924]. An investigation is in progress of the possible presence of such groups in the hormone molecule.

It is to be expected that the action of very dilute nitric acid at p_H 3 would be oxidative, and it is hoped to correlate the results now presented with those derived from a study of the effect of other oxidising agents on the hormone.

EXPERIMENTAL.

Exp. 1 (Table I). Cooled sodium nitrite solution (1.0 cc. containing the equivalent of 40 mg. of nitrous acid) was added to an ice-cold mixture of distilled water (1.0 cc.), acetic acid (3.0 cc. of 9.4*N*) and purified hormone solution (3.0 cc., containing 30 units associated with 0.25 mg. of solid). This mixture (8.0 cc. at p_H 3) was preserved in ice throughout the experiment. Samples (1.0 cc.) were withdrawn, mixed with ice-water and sodium hydroxide (0.45 cc. of 33 %), diluted to 10.0 cc. at p_H 7.4, preserved at 0° and assayed as shown in Table I. A control experiment showed that the response of the uterus to doses of oxytocic solutions of the strengths used in these tests was unaffected by sodium nitrite at a dilution comparable with that present in the assay of the first sample.

Table I. (*Exp. 1.*)

Time hours	Oxytocic activity % of initial value	Nitrous acid by starch iodide test	Stability at p_H 7.4 and 0° % of initial value
0.05	32	+	—
1	30	+	30 after 11 days
2	32	+	—
4	28	+	—
7	—	+	—
13.3	20	+	—
32	18	+	—
48	16	5.9 % of initial value*	16 after 6 days
96	—	—	—
288	18	—	—

* Colorimetrically by Ilosvay's reagent [Treadwell, 1922] using barium nitrite as standard.

Exp. 2 (Table II). Sodium nitrite solution (5.0 cc., equivalent to 200 mg. of nitrous acid) was added to a cooled mixture of water (4.0 cc.), acetic acid (15.0 cc. of 9.4*N*) and purified hormone solution (1.0 cc. containing 200 units associated with 1.95 mg. of solid). This mixture (25.0 cc.) was maintained at 3° during the experiment; the reaction remained unchanged at p_H 3. Samples, when withdrawn, were brought to p_H 7.4 and 10.0 cc. with sodium hydroxide and preserved at 0°. Estimations of nitrous acid were made by means of the Ilosvay reagent and dilutions of 0.1 cc. of the mixture.

Exp. 3. It is unnecessary to describe this experiment in detail. A mixture (9.0 cc.) of purified hormone solution (3.0 cc., containing 900 units), 9.4*N* acetic acid (5.0 cc.), and sodium nitrite solution (1.0 cc., equivalent to 400 mg. of nitrous acid) was preserved at 0° for 7 days. Samples for assay were withdrawn on the 2nd, 3rd, 5th and 7th days, and sodium nitrite solution was added on six occasions so that the concentration of nitrous acid varied between 14 and 3.6 mg. per cc. Glacial acetic acid was occasionally added to maintain the reaction at p_H 3.4–3.5. The essential results are given on p. 1220.

Table II. (*Exp. 2.*)

Time hrs. min.	With- drawn for assay	Oxytomic strength as % of total initial value	With- drawn for esti- mation of nitrous acid, cc.	Nitrous acid in volume present mg.	Nitrous acid per cc. mg.	Nitrous acid added (50 mg. per cc.) mg.	Volume
0	—	100	—	200	8	—	25.0
1	1.0	37*	—	—	—	—	24.0
15	1.0	34*	—	—	—	—	23.0
25	—	—	0.1	152	6.6	—	22.9
3 0	—	—	0.1	150	6.0	—	22.8
3 30	1.0	22	—	—	—	—	21.8
7 15	—	—	0.1	77	3.5	—	21.7
11 30	1.0	19	—	—	—	—	20.7
12 0	—	—	0.1	43	2.1	—	20.6
12 0	16.5 cc. of the reaction mixture were withdrawn and mixed with 3.5 cc. of sodium nitrite solution, equivalent to 40 mg. of nitrous acid per cc.						
12 6	—	—	—	174	8.7	—	20.0
29 0	1.5	6.6	—	—	—	—	18.5
29 0	—	—	0.1	37	2.0	—	18.4
29 0	—	—	—	—	—	85	20.1
29 0	—	—	0.1	130	6.5	—	20.0
53 0	1.5	4.2	—	—	—	—	18.5
53 0	—	—	0.1	36	2.0	—	18.4
53 0	—	—	—	—	—	85	20.1
53 0	—	—	0.1	139	6.9	—	20.0
78 0	1.5	2.2	—	—	—	—	18.5
78 30	—	—	0.1	34	1.8	—	18.4

* A direct comparison of these solutions confirmed their relative oxytomic strengths.

Exp. 4. Cooled sodium nitrite solution (1.0 cc. equivalent to 40 mg. of nitrous acid) was added to ice-cold purified hormone solution (2.0 cc., containing 60 units associated with 0.58 mg. of solid) and 9.4*N* acetic acid (3.0 cc.). The mixture (at p_H 3) was preserved in ice for 15 mins., then mixed with an aqueous glycine solution (4.0 cc., containing 320 mg. of glycine; 5 mol. to 1 mol. nitrous acid), and incubated at 37°. Preliminary experiments (not published) had shown that this treatment rapidly reduced the nitrous acid to a small concentration, and that relatively large amounts of glycine and glycollic acid did not interfere with the oxytomic assay.

Estimations were made of the concentration of nitrous acid by the Illosvay reagent; at 1½ hours this was 1.6 % of the initial value, and at 4 hours 0.003 %.

At 4½ hours, 3.0 cc. of the reaction mixture were brought to 10.0 cc. at p_H 7.2, and the remainder was left at p_H 3. 7 days later, 3.0 cc. of the remainder were brought to 10.0 cc. at p_H 7.4. All solutions were kept in ice.

The sample of 3.0 cc. taken at 4½ hours contained in all 3.57 units, whereas that taken after 7 days at p_H 3 contained 2.86 units. Since the initial content of 3.0 cc. of the experimental solution was 18 units, these values represent oxytomic strengths of 20 and 16 % of the initial value. It is uncertain whether this slight decrease is due to the presence of the residual traces of nitrous acid in the experimental mixture or to p_H -instability at p_H 3 or 7.4.

Exp. 5 (Table III). Cooled sodium nitrite solution (4.0 cc., equivalent to 42 mg. of nitrous acid per cc.) was added to a cooled mixture of glacial acetic acid (10.0 cc.) and purified hormone solution containing 3 oxytomic units per cc. This mixture was kept in ice-water during the experiment and the temperature remained fairly constantly at 5°. The initial p_H was 3; this remained unchanged, and was tested at 5, 9, 15, 22 and 30 hours. At the times shown in Table III

Table III. (*Exp. 5.*)

Time hrs. min.	Removed for assay cc.	Oxytocic value, %	Concn. of HNO ₂ , mg. per cc. of reaction mixture*	Added		Concn. of HNO ₂ , mg. per cc. of reaction mixture	Volume after treatment, cc. (estimated)
				Sodium nitrite cc.	Water cc.		
— 0	—	—	1.47	—	—	—	114.00
— 3	5.00	55	—	—	—	—	109.00
— 9	—	—	1.27	—	—	—	108.90
— 24	—	—	—	0.63	0.46	1.50	109.99
— 34	5.05	37	—	—	—	—	104.94
— 46	—	—	1.49	—	—	—	104.84
1 30	5.05	33	—	—	—	—	99.79
1 39	—	—	1.45	—	—	—	99.69
3 0	5.05	30	—	—	—	—	94.64
3 9	—	—	1.37	—	—	—	94.54
3 29	—	—	—	0.45	0.50	1.56	95.49
3 34	—	—	1.56	—	—	—	95.39
4 30	5.10	—	—	—	—	—	90.29
4 39	—	—	1.50	—	—	—	90.19
5 9	—	—	1.48	—	—	—	90.09
6 0	5.10	27	—	—	—	—	84.99
6 14	—	—	1.42	—	—	—	84.89
6 34	—	—	—	0.30	0.55	1.55	85.74
7 14	—	—	1.58	—	—	—	85.64
8 0	5.15	—	—	—	—	—	80.49
8 14	—	—	1.52	—	—	—	80.39
9 14	—	—	1.42	—	—	—	80.29
9 24	—	—	—	0.30	0.50	1.56	81.09
10 0	5.20	—	—	—	—	—	75.89
10 14	—	—	1.53	—	—	—	75.79
11 39	—	—	1.46	—	—	—	75.69
12 0	5.20	26	—	—	—	—	70.49
12 39	—	—	1.40	—	—	—	70.39
12 54	—	—	—	0.20	0.50	1.50	71.09
13 39	—	—	1.48	—	—	—	70.99
14 0	5.25	—	—	—	—	—	65.74
14 39	—	—	1.43	—	—	—	65.64
14 54	—	—	—	0.34	0.32	1.63	66.30
15 39	—	—	1.49	—	—	—	66.20
16 0	5.30	—	—	—	—	—	60.90
16 39	—	—	1.34	—	—	—	60.80
16 52	—	—	—	0.38	0.23	1.58	61.41
17 39	—	—	1.54	—	—	—	61.31
18 0	5.35	—	—	—	—	—	55.96
18 39	—	—	1.50	—	—	—	55.86
19 39	—	—	1.46	—	—	—	55.76
19 54	—	—	—	0.13	0.43	1.54	56.32
20 0	5.40	25	—	—	—	—	50.92
20 39	—	—	1.42	—	—	—	50.82
20 54	—	—	—	0.22	0.29	1.58	51.33
21 39	—	—	1.55	—	—	—	51.23
22 0	5.45	—	—	—	—	—	45.78
22 39	—	—	1.50	—	—	—	45.68
23 39	—	—	1.39	—	—	—	45.58
23 54	—	—	—	0.22	0.24	1.57	46.04
24 0	5.50	—	—	—	—	—	40.54
24 39	—	—	1.53	—	—	—	40.44
25 39	—	—	1.43	—	—	—	40.34
26 0	5.50	—	—	—	—	—	34.84
26 14	—	—	—	0.16	0.19	1.60	35.19
26 39	—	—	1.52	—	—	—	35.09
27 39	—	—	1.41	—	—	—	34.99
27 54	—	—	—	0.14	0.21	1.56	35.34
28 14	5.55	—	—	—	—	—	29.79
28 39	—	—	1.50	—	—	—	29.69
29 49	—	—	1.41	—	—	—	29.59
30 0	5.55	22	—	—	—	—	24.04

Actual volume at end of experiment 23.0 cc.

* Determined in samples of 0.1 cc. withdrawn at the times stated.

samples were withdrawn for oxytomic assay and for estimation of nitrous acid by the Illosvay method, using a barium nitrite solution as the standard. The concentration of nitrous acid was maintained at about 1.5 mg. per cc. of reaction mixture by adding the necessary amount of sodium nitrite solution as calculated from the estimation of nitrous acid. The amount withdrawn for the first sample for the oxytomic assay was 5.0 cc., but since the mixture became progressively diluted with nitrite solution, the removal of the same volume for subsequent samples would have made these relatively weaker and would thus have increased the difficulties of calculating the results of the assays. The procedure adopted was as follows: concurrently with the addition of nitrite, sufficient distilled water was added to make the total volume of liquid added equal to 1 % of the volume of the reaction mixture at the time of making the addition; the volume removed for oxytomic assay was increased by 1 % wherever an addition of nitrite had been made since the previous sample was taken. The samples for assay were diluted to 10.0 cc. at p_H 7.2-7.4.

Exp. 6 (Table IV). A mixture of purified pituitary solution (3 cc., containing 3 units per cc.) and sodium nitrate solution (1 cc., containing 86 mg., equivalent to 63 mg. of nitric acid) was adjusted to p_H 3 with a few drops of *N* acetic acid, diluted to 10.0 cc. and preserved at 0°. Samples (2 cc.) were diluted to 5.0 cc. at p_H 7.2 and preserved at 0°.

Table IV. (*Exp. 6.*)

Time hours	Days at 0° and p_H 7.2 before assay	Oxytomic value, %	Days at 0° and p_H 7.2 after assay	Subsequent oxytomic value, % (approx.)
0	—	100	—	—
22	1	89	5	60
41.5	1	82	4	30
88	2	48	—	—

At 89 hours sodium nitrite (15.4 mg., equivalent to 10 mg. of nitrous acid) was added to the residual 4 cc. of the experiment, the reaction was adjusted to p_H 3 by the addition of glacial acetic acid, and the mixture was preserved at 0° for a further period of 71 hours; free nitrous acid was then present by the starch iodide test. A sample was removed as above and assayed at once; the oxytomic value was 25 %. Several days later, when nitrous acid could no longer be detected, the oxytomic value had fallen to 20 %. This was ascertained by diluting 1.65 cc. of the reaction mixture to 4.12 cc. at p_H 7.2 and assaying at once. The oxytomic substance in this sample (regarded as being B) was shown to be stable at p_H 7.2 by preserving it at 0° for 5 days, when the activity remained unchanged at 20 %.

Experiments on the possible effect of sodium nitrate in the testing-bath showed that there was a diminution in the height of contractions when volumes of 0.7 cc. or more *M*/1000 sodium nitrate solution were present in a 100 cc. bath. This effect is presumably due to hypertonicity of the Ringer solution in the bath, since it is also produced by the addition of similar volumes of *M*/1000 sodium chloride solution.

SUMMARY.

1. When nitrous acid acts on a solution of the hormone at p_H 3, the oxytomic activity diminishes as a result of (at least) three well-defined reactions.
2. The existence of these reactions has been detected by studying the fall in activity with lapse of time when nitrous acid and nitric acid act on the hormone.

3. Nitrous acid very rapidly converts the hormone into a derivative A which has an oxytocic strength equal to 35 % of that of the hormone.
4. Nitric acid, formed by aerial oxidation of nitrous acid, then acts alone or in conjunction with nitrous acid and produces a substance B with an oxytocic strength of 20 %.
5. The prolonged action of high concentrations of nitrous acid gradually transforms B into a substance D with an oxytocic strength of about 0.1 %.
6. Nitric acid converts the hormone into a substance C (activity about 90 %), which differs from the other derivatives in being unstable at p_H 7.2. Nitrous acid transforms C into a substance which is stable at p_H 7.2 and has an activity of 20 %; this is probably identical with B.
7. It has not previously been shown that the constitution of the hormone is such as to allow its conversion into a series of derivatives which exert an oxytocic effect.

My thanks are due to Dr T. F. Macrae for his help. I am also indebted to Messrs Boots Pure Drug Company, Nottingham, who presented the whole of the posterior lobe powder used in this work.

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CLXVI. THE PROTEOLYTIC ENZYMES OF YEAST.

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(Received June 5th, 1933.)

AN investigation into the action of proteolytic enzymes on the oxytocic principle of the pituitary gland [Gulland and Macrae, 1933] required the preparation of the yeast proteolytic enzymes—dipeptidase, aminopolypeptidase and proteinase. In recent years Willstätter, Grassmann and their colleagues have described excellent methods for the preparation of these enzymes from Löwenbräu yeast, whereby complete separation of the enzymes has been achieved [1926; 1927; 1928, 1, 2, 3; 1930]. Since the strain of yeast used by these authors could not be obtained in a fresh condition, it was hoped to devise methods for the preparation of those enzymes from the more readily available English brewer's top-yeast.

It is well known that yeasts from different sources may not behave identically, and it was therefore to be expected that English and German yeasts might liberate their enzymes at different rates. Since the methods used by the German authors depended largely on the fractional autolysis of the dead yeast cells, it was improbable that their methods could be applied without modification to the preparation of the enzymes from English yeast. A brewer's top yeast was used for the greater part of the work now described, but a few experiments were carried out with a Dutch baker's yeast; Löwenbräu yeast, on the other hand, is a brewer's bottom yeast. A complete investigation of the problem has not been attempted, but the results obtained may be of value to others who may require these important enzymes.

Qualitatively the English and Dutch yeasts behaved like Löwenbräu yeast, but marked quantitative differences were observed. By modifying the method of the German authors, dipeptidase preparations were obtained containing only traces of aminopolypeptidase and completely free from proteinase. Aminopolypeptidase was prepared by a slight modification of the existing method and was completely free from dipeptidase and proteinase. The purification of proteinase, however, has offered much greater difficulties. Using English top-yeast and Dutch baker's yeast, no method gave a proteinase preparation which was free from aminopolypeptidase, although the proportion of proteinase to aminopolypeptidase was always much higher than that of normal yeast autolysates. Proteinase preparations have been obtained completely free from dipeptidase.

EXPERIMENTAL.

Methods of assaying enzyme preparations.

The procedure used for the assay of dipeptidase, aminopolypeptidase and proteinase was the method of alkali titration in 90 % alcohol [Willstätter and Waldschmidt-Leitz, 1921]. The activities of dipeptidase and aminopolypeptidase

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are estimated in units which are defined below. The activity of proteinase is estimated as the increase in acidity of a gelatin solution in unit time (see below).

The activity of dipeptidase is estimated using *dl*-leucylglycine as substrate, the unit [Willstätter and Grassmann, 1926] being that quantity of enzyme which hydrolyses one half of the *l*-peptide in 1 hour at p_H 7.8 and 40° when acting on 225 mg. of *dl*-leucylglycine in 10 cc. of *M*/30 phosphate buffer. The estimation of dipeptidase was carried out in the following manner. A solution prepared in a graduated flask (2.5 cc.) by warming 56 mg. of *dl*-leucylglycine with *M*/3 phosphate buffer at p_H 7.8 (0.25 cc.) and water (about 1 cc.) was cooled and brought to p_H 7.8 with about 2 drops of *N* ammonia. The enzyme solution was then added, and the contents of the flask diluted to the mark with water and mixed. 1.0 cc. of the mixture was immediately transferred to a flask containing absolute alcohol (9 cc.) and 4 drops of a 0.5 % alcoholic solution of thymolphthalein and titrated with *N*/20 KOH in 90 % alcohol until a faint blue colour was just perceptible. The graduated flask containing the remainder of the solution had meantime been placed in a thermostat at 40° and after 1 hour, 1.0 cc. of the solution was removed and titrated in the same way. The difference between the titration values represented the degree of hydrolysis. An increase in acidity equivalent to 0.60 cc. of *N*/20 KOH corresponds with 50 % hydrolysis of the *l*-peptide and is effected by 0.25 unit of dipeptidase. The amount of enzyme taken must be adjusted so that not less than 25 % and not more than 75 % of the *l*-peptide is hydrolysed.

The activity of aminopolypeptidase is estimated using *dl*-leucylglycylglycine as substrate, the unit [Grassmann and Dyckerhoff, 1928, 3] being that quantity of enzyme which hydrolyses one half of the *l*-peptide in 1 hour at p_H 7.0 and 40°, when acting on 245 mg. of *dl*-leucylglycylglycine in *M*/30 phosphate and *M*/25 ammonium chloride having a total volume of 10 cc. The determination of aminopolypeptidase was carried out in a similar manner to that of dipeptidase. A solution of 61 mg. of *dl*-leucylglycylglycine in *M*/3 phosphate at p_H 7.0 (0.25 cc.) and *M*/2.5 ammonium chloride-ammonia at p_H 7.0 (0.25 cc.) was adjusted to p_H 7.0 with a trace of *N* ammonia; no warming on the water-bath was required, since leucylglycylglycine is easily soluble in water. The enzyme was added and, after the solution had been diluted to 2.5 cc., 1.0 cc. portions were removed at intervals of 1 hour (temperature 40°) and titrated in the same manner as in the determination of dipeptidase. An increase in acidity equivalent to 0.50 cc. of *N*/20 potassium hydroxide corresponds with 50 % hydrolysis of the *l*-peptide and is effected by 0.25 units of aminopolypeptidase. The amount of enzyme used must effect hydrolysis of not less than 25 % and not more than 75 % of the *l*-peptide. In the presence of dipeptidase the results of the determination are too high since there is some hydrolysis of the glycylglycine formed by the action of the aminopolypeptidase.

The estimation of proteinase was carried out as follows. A mixture of a 10 % gelatin solution (6 cc.) and *M*/5 citrate buffer at p_H 5.0 (1.6 cc.) in a 10 cc. graduated flask was adjusted to p_H 5.0 with a trace of *N* acetic acid. The enzyme solution was then added, and the contents of the flask were diluted to the mark with water and immediately mixed thoroughly by being warmed in a thermostat at 40° and shaken gently. A 2 cc. portion was at once removed and added to absolute alcohol (18 cc.) which had previously been heated to 50–60°. Precipitation of gelatin occurred, and after the addition of 6 drops of thymolphthalein the mixture was titrated with *N*/20 KOH in 90 % alcohol until a permanent faint blue colour had developed; constant shaking was essential during the titration. After the hydrolysis had proceeded for 24 hours at 40°, a

further 2 cc. portion was removed and titrated. The results are quoted as increases in acidity. In presence of aminopolypeptidase high values are obtained owing to the action of that enzyme on the products of the proteinase hydrolysis of gelatin.

Dipeptidase.

Grassmann and Klenk [1930] obtained "pure" preparations of dipeptidase from Löwenbräu yeast by autolysis of the dead yeast cells for 20 hours at p_H 6.4–6.8. The autolysate thus obtained was very rich in dipeptidase and also contained some aminopolypeptidase and proteinase. Rapid adsorptions at p_H 5 with small quantities of specially prepared suspensions of aluminium hydroxide and kaolin removed all the proteinase and some of the aminopolypeptidase, but left most of the dipeptidase, which was finally obtained free from aminopolypeptidase by careful fractional precipitation with acetone. The direct application of this method to the English top-yeast invariably yielded preparations which had much dipeptidase activity, were free from proteinase, but contained some aminopolypeptidase; in the many experiments which were carried out, two different preparations of aluminium hydroxide Cy gave similar results. The weights of the dry preparations obtained were only about one half of those recorded by Grassmann and Klenk. The enzyme activities of the preparations varied to some extent, but the following were typical preparations; proteinase activity was entirely absent.

Table I.

	Units of dipeptidase per 10 mg.	Units of aminopolypeptidase per 10 mg.
1	0.48	0.20
2	0.51	0.18
3	0.62	0.23

An attempt to free one of the above preparations from aminopolypeptidase by redissolving in water and reprecipitating with acetone (compare Grassmann and Klenk, 1930) resulted in almost complete destruction of both dipeptidase and aminopolypeptidase. A preparation (500 mg., containing 0.48 unit of dipeptidase and 0.20 unit of aminopolypeptidase per 10 mg.) was dissolved in water (50 cc.) and adjusted to p_H 7.0 with ammonia and cooled to 0°. Pure acetone (30 cc.) containing sufficient ammonia to keep the final mixture at p_H 7.0 was added, and the precipitate was centrifuged down and discarded. The centrifugate was treated with a further portion of acetone (25 cc.) at 0° and the precipitate was centrifuged down and dried by treatment with acetone and finally with ether. This material (150 mg.) contained 0.05 unit of dipeptidase and 0.02 unit of aminopolypeptidase per 10 mg.

An earlier method for the preparation of solutions free from "tryptic" activity [Grassmann and Haag, 1927] depended on the removal of the enzymic impurities by adsorption with a specially prepared suspension of aluminium hydroxide. This, when applied to the English top-yeast, gave a final solution which was free from proteinase but not from aminopolypeptidase.

The behaviour of a fresh yeast autolysate towards adsorption by aluminium hydroxide Cy was investigated, in order to discover if a separation of dipeptidase and aminopolypeptidase could be effected by this means. English top-yeast (200 g.) was liquefied with ethyl acetate (20 cc.) and treated with water (220 cc.). Dilute ammonia was added to neutralise the acid as it was formed in the autolysis, and to maintain the p_H at 6.5. After 2 hours of this treatment

the yeast was centrifuged and carefully washed with water. It was now suspended in water (250 cc.) containing a few drops of toluene and allowed to autolyse for 17 hours, the p_H being maintained between 6.2 and 6.5. The yeast was centrifuged, and portions of 25 cc. each of the centrifugate were immediately cooled to 0° , adjusted to p_H 5.0 with *N* acetic acid, mixed with *M* sodium acetate-acetic acid buffer at p_H 5.0 (2 cc.) and treated in the following manner with a suspension of aluminium hydroxide *Cy* (35 mg. Al_2O_3), the total volume being 35 cc. The adsorptions were carried out as quickly as possible and the temperature was kept below 10° . One portion was treated once with the adsorbent, another portion twice, and a third portion three times successively, the same amount of adsorbent being used in each case. The dipeptidase and aminopolypeptidase activities of the original autolysate and the three adsorbed portions were estimated (Table II).

Table II.

	Units of dipeptidase in solution representing 1 cc. of original autolysate	Units of aminopoly- peptidase in solution representing 1 cc. of original autolysate
Original autolysate	3.35	1.80
After one adsorption	1.10	1.25
After two adsorptions	0.25	0.45
After three adsorptions	0.16	0.11

It is apparent from this experiment that simple adsorption with aluminium hydroxide *Cy* under these conditions does not suffice for the separation of dipeptidase and aminopolypeptidase. Grassmann and Klenk [1930], using very similar conditions to those described above, observed that aminopolypeptidase was preferentially adsorbed, whereas in this experiment preferential adsorption of dipeptidase occurred. It would appear, therefore, that a quantitative difference exists between the adsorptive properties of the enzymes in autolysates of Löwenbräu yeast and English top-yeast.

Many attempts were made to obtain "pure" dipeptidase preparations. The following method, which is a modification of that of Grassmann and Klenk [1930], was found to be the most successful. English top-yeast (500 g.) was liquefied with ethyl acetate (50 cc.) and treated with water (500 cc.). 20 % ammonia was added carefully in order to maintain the suspension at p_H 6.5. After 2 hours the yeast was centrifuged and the dark brown centrifugate set aside. The yeast was carefully washed with water, again suspended in water (500 cc.) containing some toluene, allowed to autolyse for 20 hours at p_H 6.2-6.6 and room temperature and centrifuged off. Meantime an aluminium hydroxide suspension had been prepared [compare Grassmann and Haag, 1927]. A quantity (400 cc.) of the dark brown centrifugate which had been retained was diluted to 1600 cc. with water and adjusted to p_H 5.0 with *M* acetic acid. A suspension of aluminium hydroxide *Cy* (700 mg. Al_2O_3) was added at 0° and after shaking the aluminium hydroxide was centrifuged down. This aluminium hydroxide which had thus adsorbed considerable amounts of autolysis products was now suspended in water and made up to 50 cc. Immediately after centrifuging from the yeast a portion of the autolysate (100 cc.) was adjusted to p_H 5 with acetic acid and mixed with *M* sodium acetate-acetic acid buffer at p_H 5.0 (2 cc.). This mixture was treated as quickly as possible four successive times at 0° with 10 cc. portions of the above aluminium hydroxide suspension. The residual enzyme solution

was then treated twice at 0° with successive portions (20 cc.) of a kaolin suspension (acid to phenolphthalein and alkaline to methyl red), containing 300 mg. of kaolin per cc. The kaolin was discarded, and a portion of this solution (120 cc.) was adjusted to p_H 7.0 with *N* ammonia, cooled to 0° and mixed with pure acetone (85 cc.) which contained sufficient ammonia to maintain the p_H at 7.0 (determined by previous trial on test portions). The resulting precipitate was centrifuged off and discarded. The centrifugate was treated with acetone (60 cc.) in the same way, and the precipitate was centrifuged and dried by washing three times with acetone, three times with ether and finally in a vacuum desiccator; yield 90 mg. This preparation contained 0.24 unit of dipeptidase and 0.005 unit of aminopolypeptidase per 10 mg. and was completely free from proteinase. After 2 months its activity remained unchanged.

This method differs from that of the German authors at several points. The most important deviation is in the larger volume of acetone used in precipitating the discarded solid of the first acetone treatment, and it is on this modification that the success of the method depends. The weight of the dry preparation and its enzymic content were very much less than those obtained by Grassmann and Klenk.

Aminopolypeptidase.

The method of Grassmann and Dyckerhoff [1928, 3] for the preparation of "pure" aminopolypeptidase from Löwenbräu yeast depends on the fact that the autolysis of the dead yeast cells in 0.3 % ammonia for 48 hours or less results in the liberation of all the proteinase activity and the destruction of all the dipeptidase activity. On the other hand, only part of the aminopolypeptidase is liberated during that time. Therefore, by centrifuging and washing the yeast after an autolysis lasting 48 hours, and by allowing it to autolyse in fresh water for a further 5 days, these authors obtained an autolysate containing aminopolypeptidase, but free from dipeptidase and proteinase. From this a dry preparation was obtained. On following this method with English top-yeast, the final preparation always contained traces of dipeptidase, but the modification described below yielded preparations free from both proteinase and dipeptidase.

English top-yeast (500 g.) was liquefied with chloroform (50 cc.) and treated with 0.35 % ammonia (500 cc.). After remaining at room temperature for 48 hours the yeast was centrifuged and thoroughly washed with water. It was then suspended in water (400 cc.) containing a little chloroform, and adjusted to p_H 7.0–7.2. After remaining for 5 days at room temperature the yeast was centrifuged off, and the centrifugate was treated with 2.5 *N* acetic acid (65 cc.). The precipitate was centrifuged, suspended in water (20 cc.) and treated with drops of *N* ammonia until all but a trace of insoluble matter had dissolved (p_H 7.5). The solution, centrifuged from the solid, was dialysed for 14 hours in a cellophane bag against a slow stream of distilled water. The enzyme solution, cooled to 0°, was now added to three times its volume of pure acetone which had been cooled to –15°. The temperature of the mixture was –8°. The precipitate was centrifuged immediately and dried rapidly by washing three times with acetone, three times with ether and finally in a vacuum desiccator; yield 165 mg. This preparation contained 0.64 unit of aminopolypeptidase per 10 mg. and was completely free from dipeptidase and proteinase. When kept in a tightly stoppered tube its activity remained unaltered for 3 months.

Proteinase.

Grassmann and Dyckerhoff [1928, 3] obtained solutions of proteinase which were free from dipeptidase and aminopolypeptidase by making use of the following facts. Dipeptidase is destroyed during a preliminary autolysis of Löwenbräu yeast in 0.3 % ammonia lasting 17 hours. If the yeast be centrifuged, washed and allowed to autolyse in fresh water for a further 5 hours at p_H 5, the liberation of aminopolypeptidase is completely suppressed, whilst the proteinase enters the autolysate. The enzyme solution prepared in this way was adjusted to p_H 8.5 in order to precipitate inorganic phosphate, which was centrifuged off. After the reaction of the solution had been adjusted to p_H 5 the proteinase was adsorbed by treatment with aluminium hydroxide $C\gamma$ and eluted with diammonium phosphate. The German authors observed that the resulting proteinase solution underwent self-activation when preserved for 10 days at 0° and p_H 7.0, and they thus obtained a highly active proteinase solution, which was completely free from dipeptidase and aminopolypeptidase.

In the present investigation the following attempts were made to obtain pure proteinase preparations from English top-yeast and Dutch baker's yeast. In each case the final preparation was preserved at p_H 7.0 and 0° for 10 days before the enzymes were estimated (Table III) in order to allow the activator to develop.

(a) The method of Grassmann and Dyckerhoff [1928, 3] was carefully followed using English top-yeast. The purification by adsorption on aluminium hydroxide was carried out.

(b) As (a) using Dutch baker's yeast.

(c) The solution from (b) was adjusted to p_H 10 with 5*N* ammonia and maintained at that p_H at room temperature for 1 hour. It was then adjusted to p_H 7.0 with 5*N* acetic acid.

(d) As in (c), but the enzyme solution was maintained at p_H 10 for 3 hours.

(e) Using English top-yeast, the method of Grassmann and Dyckerhoff [1928, 3] was modified by carrying out the second autolysis at p_H 4.6 instead of 5.0.

(f) and (g). Procedure as in (a), except that the duration of the preliminary autolysis was reduced from 17 hours to 12 hours. In (f) the duration of the second autolysis was 3 hours, in (g) 5 hours.

(h) A portion (75 cc.) of the autolysate prepared from English top-yeast according to Grassmann and Dyckerhoff [1928, 3, p. 62] was treated with pure acetone (150 cc.) at room temperature. The precipitate was centrifuged off and redissolved in water (25 cc.). Acetone (30 cc.) was then added at room temperature, and the precipitate was centrifuged off and dried by washing with acetone, then ether and finally in a vacuum desiccator; yield 25 mg.

(i) A portion (75 cc.) of the same enzyme solution as that used in (h) was dialysed in a cellophane bag against running tap-water for 40 hours and then against a slow stream of distilled water for 6 hours. The solution was then mixed with acetone (100 cc.) at room temperature, and the precipitate was centrifuged off and dried as in (h); yield 35 mg.

The activities of the above preparation are recorded in Table III. All the preparations contained aminopolypeptidase and since that enzyme hydrolyses further the products of the proteinase hydrolysis of gelatin, no accurate figure for the increase in acidity due to the proteinase action could be obtained. However, the recorded increases in acidity give strong indication of the proteinase contents of the preparations. As already explained the figures for amino-

polypeptidase are also inaccurate in those preparations which contain dipeptidase. Complete hydrolysis of the *l*-leucylglycylglycine was obtained in the determination of the aminopolypeptidase activity of some of the preparations; this requires an increase in acidity of 1.00 cc. of *N*/20 KOH hydroxide.

Table III.

	Proteinase	Aminopolypeptidase	Dipeptidase
	In (a)-(g), 1 cc. of enzyme solution in a total volume of 10 cc. In (h) and (i), 10 mg. of dry preparation in 10 cc.	In (a)-(g), 2 cc. of enzyme solution in a total volume of 10 cc. In (h) and (i), 20 mg. of dry preparation in 10 cc.	In (a)-(g), 2 cc. of enzyme solution in a total volume of 10 cc. In (h) and (i), 20 mg. of dry preparation in 10 cc.
	Increase in acidity in 2 cc. portions after 24 hours. cc. <i>N</i> /20 KOH	Increase in acidity in 1 cc. portions after 24 hours. cc. <i>N</i> /20 KOH	Increase in acidity in 1 cc. portions after 24 hours. cc. <i>N</i> /20 KOH
(a)	1.10	1.01	0.05
(b)	1.60	0.96	0.09
(c)	1.33	0.83	0.01
(d)	0.97	0.56	0.00
(e)	0.46	0.88	Not determined
(f)	0.78	1.06	0.45
(g)	1.31	0.99	0.71
(h)	1.35	1.00	0.40
(i)	1.30	0.89	0.00

The results of these attempts to obtain a pure proteinase are here summarised. Using English top-yeast and Dutch baker's yeast the method of Grassmann and Dyckerhoff [1928, 3] yielded a preparation which contained considerable quantities of aminopolypeptidase and traces of dipeptidase (*a* and *b*). Treatment with ammonia readily destroyed these traces of dipeptidase, giving enzyme preparations which were completely free from dipeptidase but still contained aminopolypeptidase (*c* and *d*). Evidently in the case of these yeasts the liberation of aminopolypeptidase at p_H 5.0 is not completely suppressed; even at p_H 4.6 considerable quantities of aminopolypeptidase were liberated although less than half of the proteinase obtained at p_H 5.0 was set free (*e*).

Löwenbräu yeast liberates its proteinase only after about 16 hours' autolysis [Grassmann and Dyckerhoff, 1928, 3]. English top-yeast begins to liberate its proteinase much sooner, for a preliminary autolysis of 12 hours and a subsequent autolysis of 3 hours at p_H 5.0 yielded considerable quantities of the three proteolytic enzymes in the second autolysate (*f*). When the second autolysis was continued for 5 hours the amounts of the enzymes liberated had increased (*g*).

Grassmann and Dyckerhoff [1928, 1, 2] observed that precipitation of enzymic solutions with acetone at room temperature destroyed most of the aminopolypeptidase. In the case of the autolysates from English top-yeast, however, two successive precipitations did not destroy all the aminopolypeptidase activity. Moreover, some dipeptidase also persisted (*h*). Prolonged dialysis also destroyed aminopolypeptidase [Grassmann and Dyckerhoff, 1928, 1, 2]. Dialysis for 46 hours of the autolysate from English top-yeast followed by acetone precipitation yielded a dry preparation which still contained aminopolypeptidase but was free from dipeptidase (*i*).

SUMMARY.

1. The liberation of the proteolytic enzymes, dipeptidase, aminopolypeptidase and proteinase by the autolysis of English top-yeast and Dutch baker's yeast has been studied and has been shown to differ in several respects from the

liberation of these enzymes from Löwenbräu yeast. Quantitative differences in the behaviour of the enzymes from these sources to adsorbents and other treatment have also been observed.

2. The methods used by Willstätter, Grassmann and their colleagues for the preparation of dipeptidase, aminopolypeptidase and proteinase from Löwenbräu yeast require modification when used for the preparation of these enzymes from English top-yeast.

3. By means of modifications of these methods, preparations of (a) dipeptidase completely free from proteinase and containing only traces of aminopolypeptidase, and (b) aminopolypeptidase free from both dipeptidase and proteinase have been obtained from English top-yeast.

4. Proteinase, free from dipeptidase but not from aminopolypeptidase, has been obtained from English top-yeast and Dutch baker's yeast.

I wish to express my thanks to Dr J. M. Gulland for his continued criticism and advice. My thanks are also due to Messrs Watney, Combe and Reid, who supplied the English top-yeast.

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CLXVII. THE OXYTOMIC HORMONE OF THE POSTERIOR LOBE OF THE PITUITARY GLAND.

III. THE ACTION OF PREPARATIONS OF PLANT PROTEOLYTIC ENZYMES.

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(Received June 29th, 1933.)

It was recorded by Dudley [1919] that trypsin, in the form of Merck's pancreatin in *N*/10 sodium carbonate solution, destroyed the oxytomic hormone. Later, Dale and Dudley [1921] observed that the hormone was inactivated by intestinal erepsin, in the form of a crude preparation from dog's intestinal mucosa in *N*/10 sodium carbonate solution. They also found that a papain preparation, which could digest fibrin in *N*/10 hydrochloric acid solution, did not attack the hormone under comparable conditions. Thorpe [1926] confirmed the destructive action at about p_H 9 of pancreatic trypsin, which had been freed from accompanying lipase but was otherwise crude. Freudenberg *et al.* [1932] stated briefly that the hormone was not altered by pepsin or erepsin but was destroyed by trypsin and papain. Dudley [1923] and also Thorpe concluded that their results indicated the presence of a peptide linkage in the hormone molecule, and Freudenberg *et al.* considered that the hormone has either a high molecular weight or is bound to a high-molecular carrier of the protein group.

The experiments of the earlier investigators were made at a time when the need for controlling hydrogen ion concentration and for using "pure" enzymes was not fully realised. For these reasons, and in view of the discrepancy between the results mentioned above and the importance of the conclusions reached, if these are accurate, it was essential to extend these experiments on a more elaborate scale using enzyme preparations of known purity or content².

The yeast proteolytic enzymes which have been studied by the Willstätter school [for summary, see Grassmann, 1932] are more easily obtained in greater purity than are animal proteolytic enzymes; in addition, they are more specific and might therefore yield more detailed information as to the nature of the hormone. It was decided to study in the first instance the action on the hormone of the dipeptidase, aminopolypeptidase and proteinase of yeast. This communication also includes a description of the action of a papain preparation. A subsequent memoir will describe the behaviour of the hormone when acted on by preparations of trypsin and other enzymes: these experiments have been completed.

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² This work was initiated in January 1932 as part of the general scheme for this series of investigations which was begun in Part II [Gulland, 1933]; a preliminary account of some of the results has already been published [Gulland and Macrae, 1933].

The hormone was slowly inactivated by two aminopolypeptidase preparations from different sources (Fig. 1). Neither preparation contained any dipeptidase or proteinase activity.

The hormone was very rapidly inactivated by two dipeptidase preparations from different sources; both were free from proteinase but contained traces of aminopolypeptidase (Fig. 2).

The hormone was very rapidly inactivated by two solutions of proteinase which contained some aminopolypeptidase (Fig. 3). One solution also contained traces of dipeptidase, whilst the other was free from this enzyme.

These results showed that the inactivation of the hormone was not caused by dipeptidase, aminopolypeptidase or proteinase for the following reasons.

(i) The inactivation was not due to dipeptidase, because the hormone was rapidly inactivated by a proteinase solution which contained no dipeptidase.

(ii) The inactivating enzyme was not proteinase, because the hormone was rapidly inactivated by a dipeptidase preparation which was free from proteinase.

(iii) Aminopolypeptidase was not responsible, because dipeptidase preparations which contained only a trace of aminopolypeptidase were many times more active than were aminopolypeptidase preparations of high aminopolypeptidase activity.

The shapes of the p_H -activity curves (Fig. 4) of the inactivating enzymes of dipeptidase and proteinase preparations were identical and had a p_H -optimum at 7.4. It was therefore highly probable that the inactivation in each case was brought about by one and the same enzyme, and this assumption is now made.

The hormone was extremely rapidly inactivated by a papain preparation, and the shape of the p_H -activity curve and position of the p_H -optimum (Fig. 4) were the same as those of the enzyme of the yeast preparations. It is presumed, therefore, that the enzyme present in the papain preparation, which inactivated the hormone, is not a papainase but is identical with the yeast enzyme.

The p_H -activity curve of this enzyme which inactivates the hormone is quite distinct from those of yeast dipeptidase, aminopolypeptidase, proteinase and papain (Fig. 5). Experiments made in the search for the substrate characteristic of this enzyme will be described in future communications, together with observations on the conclusions of other investigators which were mentioned above.

It is noteworthy that none of the enzyme preparations reduced the oxytocic activity to zero. For example, a dipeptidase preparation reduced the oxytocic strength to 0.7 % of its initial value in half an hour, but no further decrease occurred in the course of 24 hours. It has been demonstrated that this residual activity is not due to histamine, of which small traces might conceivably have occurred in the stock hormone solution. Our present opinion is that this residual activity is due to a derivative of the hormone produced by the action of the enzyme.

EXPERIMENTAL.

Methods employed.

Preparation of yeast proteolytic enzymes. Dr W. Grassmann of Munich presented us with (1) a dipeptidase preparation which was free from proteinase but contained traces of aminopolypeptidase; (2) an aminopolypeptidase preparation which contained neither dipeptidase nor proteinase.

Our dipeptidase and aminopolypeptidase preparations were made from English brewer's top yeast, our proteinase solutions from Dutch baker's yeast, in each case as described by Macrae [1933].

Estimation of proteolytic activity. The activity of the proteolytic enzymes was estimated in the usual way by comparing the volumes of $M/20$ alcoholic KOH required to titrate to thymolphthalein standard portions removed from the enzyme-substrate mixture at the beginning of the estimation and after a known period of time. The amounts of dipeptidase and aminopolypeptidase were then expressed in units [Willstätter and Grassmann, 1926; Grassmann and Dyckerhoff, 1928].

Assay of oxytomic activity. The oxytomic activity of solutions was assayed by the degree of contraction they produced in the isolated uterus of the guinea-pig [Burn and Dale, 1922; Burn, 1928; Gulland and Newton, 1932].

In each time-activity experiment dilutions of the stock solutions were used as standards, the results of the assays being expressed as percentages of the initial activity of the stock solution (regarded as 100). In p_H -activity experiments the activities of the control solutions were assayed against a dilution of the stock solution, and the activities of the samples were then assayed against their control solutions (regarded as 100). For the sake of clarity the activity in international standard units of the stock solution is quoted for each experiment.

Samples and controls. Samples withdrawn for assay or as controls were adjusted to p_H 3.5 with acetic acid, being diluted with water to a known volume which was much too concentrated in oxytomic material to be used directly for assay. These stock samples were preserved at 0° , and suitable dilutions of them for assay were adjusted to p_H 7.5 immediately before use and were kept at 0° until no longer needed. By this means the hormone present in a sample was stored almost at the p_H of maximum stability (p_H 3 [Gaddum, 1930]), but was pipetted into the testing-bath at a reaction which did not upset the normal buffering of the Ringer solution, without being exposed unnecessarily to the deteriorating influence of an alkalinity of p_H 7.5. The selection of p_H 3.5 instead of p_H 3 was made because experience showed that the amount of acetic acid required to change the reaction to p_H 3.5 was relatively small as compared with that needed to carry the adjustment further to p_H 3. The assumption has necessarily been made that the final product (0.7 % activity) of the action of the enzyme which destroys the hormone is stable at p_H 3.5 and at p_H 7.5 under the conditions of the experiments.

It should be noted that "boiled enzyme controls" have been avoided. The rate of destruction of the hormone is dependent on the hydrogen ion concentration and is a function of the temperature [Gaddum, 1930]; it was therefore essential that a control solution should be an accurate control of the effect of heat and reaction on the hormone under the same conditions as obtained in the experiment. A further reason for avoiding "boiled enzyme controls" was the fact that many enzyme solutions deposit amorphous precipitates of denatured proteins when heated, and these might adsorb the hormone. This difficulty, however, has not been encountered.

Action of yeast aminopolypeptidase preparations.

(A) *From English brewer's top yeast, at p_H 7.0.* A hormone solution (16.7 cc. at p_H 3, containing 4.5 international oxytomic units per cc.), $M/3$ sodium phosphate buffer at p_H 7.0 (2.5 cc.), and $M/2.5$ ammonium chloride solution (2.5 cc.) were mixed in a graduated flask (25 cc.), and the reaction was adjusted to p_H 7.0 with N ammonia. A solution of an aminopolypeptidase preparation (100 mg. in 2.5 cc. of water, containing 3.2 units of aminopolypeptidase and free from dipeptidase and proteinase) was added, and the mixture was diluted as quickly as possible to 25.0 cc. with water and thoroughly mixed. A control

sample (1.5 cc.) was immediately removed into a graduated flask (2.5 cc.), which was at once immersed for 5 minutes in a vigorously boiling water-bath in order to inactivate the enzyme. When this heating was finished, the flask was tightly stoppered and placed in a thermostat at 40°. As soon as the control sample had been removed from the experimental solution, the flask containing the remainder of this solution was tightly stoppered and placed in the thermostat.

Sample portions (1.5 cc. each) were removed from the reaction mixture into 2.5 cc. flasks at appropriate intervals; each was heated at 100° for 5 minutes to inactivate the enzyme immediately after being removed, and the flask was then stoppered and returned to the thermostat. At the end of the experiment, 2.5 *M* acetic acid (0.5 cc.) was added to each sample and the control, and the mixtures were diluted with water to 2.5 cc.; the p_H was then 3.5, and these stock samples were preserved at 0°.

The reaction remained constant at p_H 7.0 during the experiment. The aminopolypeptidase present in the residual solution after 24 hours was estimated, using *dl*-leucylglycylglycine as substrate. 3.0 cc. contained 0.35 enzyme unit, whereas initially 3.0 cc. had contained 0.38 unit, so that more than 90 % of the aminopolypeptidase was present at the end of the experiment.

For the oxytocic assay 1.0 cc. of each stock sample was diluted to 10.0 cc. at p_H 7.5 and tested against a dilution of the stock hormone solution (0.4 cc. in 10 cc., *i.e.* 0.18 unit per cc.). These solutions would have had the same oxytocic activity if no inactivation of the hormone had occurred. The results are given in Table I and Fig. 1, *A*.

Table I.

Time (hours)	0	0.5	1	1.5	2	3	4	8	24
Activity present (%)	88	76	67	48	40	29	23	8	2.5

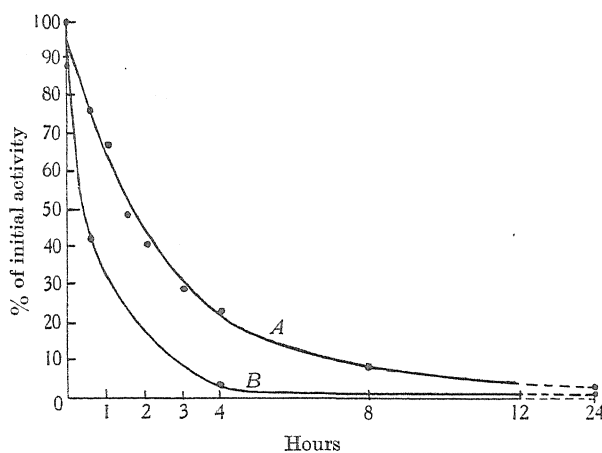


Fig. 1. Aminopolypeptidase preparations: *A*, from English yeast; *B*, from Munich yeast.

(*B*) *From Munich yeast, at p_H 7.5.* An aminopolypeptidase preparation (20 mg., containing 0.86 unit of aminopolypeptidase, no proteinase and no dipeptidase) was dissolved in *M*/3 sodium phosphate buffer at p_H 7.5 (2.0 cc.) and added to a hormone solution (8 cc., containing 1.5 units per cc.), previously adjusted to p_H 7.5. The mixture was shaken, a control portion (2.0 cc.) was immediately removed into a graduated flask (2.5 cc.), and the residual solution

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was incubated at 40°; the p_H remained constant at 7.5. The technique described above was used for control and samples, and at the end of the experiment these were diluted to 2.5 cc. (p_H 3.5) with 2.5 *M* acetic acid.

For assay the stock samples were diluted (1.88 cc. to 10.0 cc. at p_H 7.5) with water and alkali and tested against a dilution of the stock hormone solution (1.2 cc. diluted to 10.0 cc., i.e. 0.18 unit per cc.). The results are given in Table II and Fig. 1, *B*.

Table II.

Time (hours)	0	1	4	24
Activity present (%)	100	42	3.7	About 1

Action of yeast dipeptidase preparations.

(*A*) From *English brewer's top yeast*, at p_H 7.8. A dipeptidase preparation (57 mg., containing 1.37 units of dipeptidase, 0.03 unit of aminopolypeptidase and no proteinase) was dissolved in water (1 cc.) containing a few drops of *M*/3 sodium phosphate buffer at p_H 7.8 and added to a mixture of a hormone solution at p_H 7.8 (6.7 cc., containing 4.5 units per cc.) and *M*/3 sodium phosphate buffer at p_H 7.8 (1 cc.). The reaction mixture was rapidly diluted to 10.0 cc. and mixed. Control and sample portions (1.5 cc. each) were removed into 2.5 cc. graduated flasks, the procedure being as described above. The p_H remained constant at 7.8 throughout the experiment, and after 24 hours 1 cc. of the reaction mixture contained 0.05 unit of dipeptidase when estimated by its power of hydrolysing *dl*-leucylglycine; this corresponded to about 40 % of the original dipeptidase activity.

Table III.

Time (hours)	0	0.5	1	4	24
Activity present (%)	80	4	About 2	About 2	About 2

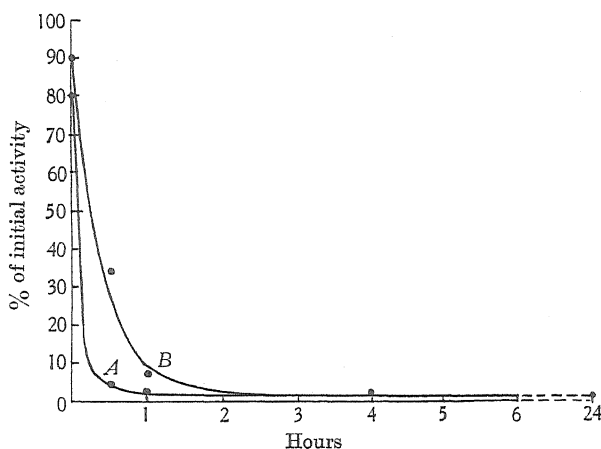


Fig. 2. Dipeptidase preparations: *A*, from English yeast; *B*, from Munich yeast.

For assay the stock samples were diluted (1.0 cc. to 10.0 cc. at p_H 7.5) and compared with a dilution of the stock hormone solution (0.4 cc. to 10.0 cc.; i.e. 0.18 unit per cc.); these solutions would have had the same oxytocic activity if no inactivation had occurred. The results are given in Table III and Fig. 2, *A*.

(B) *From Munich yeast, at p_H 7.5.* A dipeptidase preparation (10 mg., containing 0.8 unit of dipeptidase, 0.02 unit of aminopolypeptidase and no proteinase) was dissolved in $M/3$ sodium phosphate buffer at p_H 7.5 (1 cc.) and added to a hormone solution at p_H 7.5 (0.5 cc., containing 200 units per cc.). The mixture was at once diluted to 5 cc. and mixed. Control and sample portions (1.0 cc. each) were removed into 2.5 cc. flasks, the procedure being as described above. The p_H remained constant at 7.5.

For assay a portion of each stock sample (0.25 cc.) was diluted to 10.0 cc. at p_H 7.5. These solutions would have been equivalent to 0.2 unit per cc. if no inactivation had occurred, and were compared with a thousand-fold dilution of the stock hormone solution. The results are given in Table IV and Fig. 2, B.

Table IV.

Time (hours)	0	0.5	1	4
Activity present (%)	90	34	7	0.8

The action of yeast proteinase preparations.

The enzyme solution was prepared from Dutch baker's yeast and contained considerable quantities of proteinase, some aminopolypeptidase and traces of dipeptidase (Table V). A second solution was prepared by adjusting the reaction of part of the first solution to p_H 10 with ammonia, maintaining it thus for 2 hours at 18° and then re-adjusting the p_H to 7; this destroyed the dipeptidase and reduced the proteinase and aminopolypeptidase activities (Table V).

Table V.

The conditions for the estimations were those customarily used for these enzymes.

The figures show the proteolytic activity before and after treatment at p_H 10.

Enzymes	Dipeptidase	Aminopoly-peptidase	Proteinase
Proteinase solution	0.5 cc. diluted to 2.5 cc.	0.5 cc. diluted to 2.5 cc.	1.0 cc. diluted to 5.0 cc.
Substrate	<i>d</i> -Leucyl-glycine	<i>d</i> -Leucylglycyl-glycine	Gelatin
Increase in activity of 1 cc. portions after 20 hrs. (cc. $N/20$ KOH)	Before 0.01 After 0.00	Before 0.83 After 0.60	Before 0.65 After 0.35

Experiments on the inactivation of the hormone were carried out with these enzyme solutions at p_H 7 and at p_H 5, the normal optimum of yeast proteinase. The procedure was identical in each case. At p_H 5 buffering was effected by $M/50$ sodium citrate buffer and at p_H 7 by $M/30$ sodium phosphate. The hormone solution (6.7 cc. containing 4.5 units per cc.), buffer solution (1 cc. of $M/5$ citrate or $M/3$ phosphate) and enzyme solution (2 cc.) were mixed and diluted with water to 10.0 cc. at the required p_H (5 or 7). A control sample (1.5 cc.) was instantly removed, heated for 3 minutes in a boiling water-bath and placed in a thermostat at 40°, in which the remainder of the reaction mixture had meantime been immersed. Further samples (1.5 cc. each) were removed at the same intervals in each case and were diluted to 2.5 cc. at p_H 3.5 as usual.

The reaction mixtures of the experiments at p_H 5 were shown to contain after 24 hours an amount of proteinase comparable with that present at the beginning of the experiments; an accurate estimation was impossible, but more than 75 % was present.

OXYTOCIC HORMONE AND YEAST PROTEOLYTIC ENZYMES 1243

The oxytocic assays were all made by comparing the activities of dilutions of the stock samples (1 cc. to 10 cc.) at p_H 7.5 with a dilution of the stock hormone solution (0.4 cc. to 10 cc.); the activities of these dilutions would have been the same (0.18 unit per cc.) if no inactivation had occurred. The results are given in Table VI and Fig. 3.

Table VI.

Time hours	Enzyme before treatment at p_H 10		Enzyme after treatment at p_H 10	
	At p_H 5	At p_H 7	At p_H 5	At p_H 7
Control	100	82	100	86
1	60	1.6	82	2.2
4	7.5	About 1	60	About 1
24	1.5	About 1	8	About 1

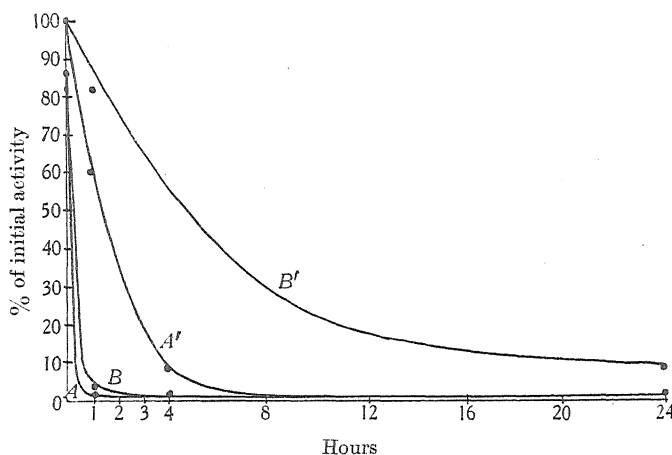


Fig. 3. Proteinase solutions: *A* and *A'*, enzyme solution before partial inactivation at p_H 10. *B* and *B'*, enzyme solution after partial inactivation at p_H 10; *A* and *B*, exp. at p_H 7; *A'* and *B'*, exp. at p_H 5.

The yeast enzyme which inactivates the hormone has a stability at p_H 10 comparable with those of aminopolypeptidase and proteinase, but it is more stable than dipeptidase.

Action of a papain preparation.

The enzyme material used was a British Drug Houses' preparation of papain, the proteolytic activity of which was determined as follows. 10 mg. of the preparation acting in the absence of hydrogen cyanide on gelatin in 10 cc. of solution at p_H 5 under the conditions of yeast proteinase estimations caused an increase in acidity in 5 hours corresponding to 0.84 cc. of $N/20$ KOH for each sample of 1 cc.

A solution of this papain preparation (20 mg.) in $M/3$ sodium phosphate buffer at p_H 7.5 (2 cc.) was added to a hormone solution (8 cc.) containing 1.5 units per cc. A control sample (2.0 cc.) was immediately removed, the remainder of the experiment being carried out in accordance with the usual procedure; all samples were diluted to 2.5 cc. at p_H 3.5. The p_H remained constant at 7.5.

For assay a dilution of each sample (1.88 cc. in 10 cc.) was compared with a dilution of the stock hormone solution (1.2 cc. in 10 cc.); these solutions

would have had the same activity (0.18 unit per cc.). The results are given in Table VII.

Table VII.

Time (hours)	0	1	4	24
Activity present (%)	40*	About 1	About 1	About 1

* Very rapid inactivation had taken place owing to the high activity of the enzyme preparation.

Effect of hydrogen ion concentration on the rate of inactivation of the hormone.

(A) *Dipeptidase preparation from English brewer's top yeast.* A solution of the enzyme (2.5 mg., containing 0.13 unit of dipeptidase, 0.04 unit of aminopolypeptidase and no proteinase) in water (0.5 cc.) was added to a mixture of hormone solution (0.5 cc., containing 76.8 units per cc.) and $M/3$ sodium phosphate buffer at the required reaction in a graduated flask (2.5 cc.). After the contents of the flask had been mixed, a control sample (1.0 cc.) was immediately removed into a graduated flask (2.5 cc.), which was immersed in a vigorously boiling water-bath for 3 minutes to destroy the enzyme, cooled to about 40° and placed in a thermostat at 40°. The remainder of the experimental solution had meantime been placed in the thermostat, and after 20 minutes a sample was removed and treated as described above. The reaction of the liquid remaining in the flask was then determined, and this value taken as the p_H of the experiment; in practice, this seldom varied from the initial reaction. The samples were then removed from the thermostat, diluted to 2.5 cc. with 2.5 M acetic acid and water (finally at p_H 3.5) and preserved at 0° for assay.

Experiments were carried out at p_H 5.7, 6.5, 8.3 and 9.5. Samples were assayed against the corresponding control solutions, 0.52 cc. of each being diluted to 20 cc. at p_H 7.5; this dilution would have contained 0.16 unit per cc. Several control solutions were assayed against a dilution of the stock hormone solution and were shown to be unaltered or to have suffered only slight inactivation. The results are given in Table VIII and Fig. 4.

Table VIII.

p_H	5.7	6.5	7.5	8.3	9.5
Inactivation (%)	15	29	39	29	14

(B) *Proteinase solution from Dutch yeast.* 1.0 cc. of the proteinase solution used contained less than 0.005 unit of dipeptidase, 0.05 unit of aminopolypeptidase and that quantity of proteinase which in 19 hours caused an increase in the acidity of a 1.0 cc. sample corresponding to 0.85 cc. of $N/20$ KOH, when acting on gelatin in a volume of 2.5 cc. of mixture under the conditions of the proteinase estimation.

The proteinase solution (1.0 cc.) at the required reaction was added to a hormone solution (1.0 cc., containing 38.4 units per cc.) which had been adjusted to the required p_H . The mixture was rapidly diluted to 2.5 cc., and a control sample (1.0 cc.) was removed. The procedure in these experiments was that described in the case of the dipeptidase preparation, except that the samples were incubated for 30 instead of 20 minutes. Experiments were carried out at p_H 5.2, 6.0, 7.0, 8.0 and 9.0.

Each control sample was assayed against a dilution of the stock hormone solution containing 0.16 unit per cc., the control solution (0.52 cc.) being diluted to 20 cc. at p_H 7.5 (0.16 unit per cc. for no inactivation). Each sample was assayed against its control sample. The results are given in Table IX and Fig. 4.

(C) *Papain preparation, B.D.H.* The papain preparation (2.5 mg., having the same activity as that described on p. 1243), dissolved in water (0.5 cc.), was added to a mixture of a hormone solution (0.5 cc., containing 156 units per cc.) and *M*/3 sodium phosphate buffer (1.5 cc.) at the required reaction.

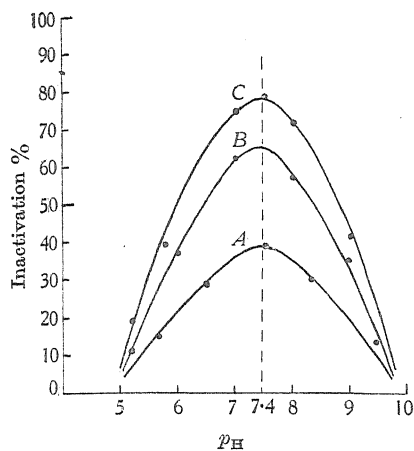


Fig. 4.

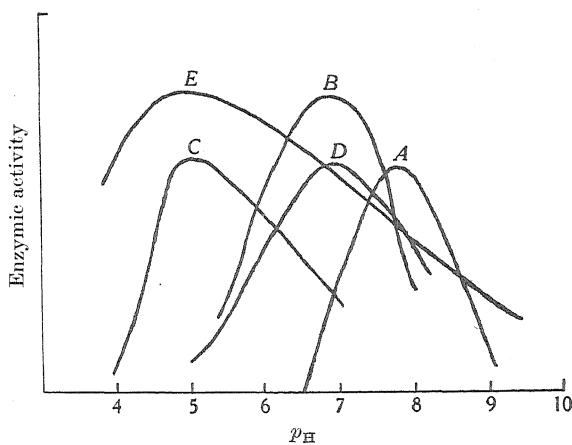


Fig. 5.

Fig. 4. Enzyme preparations: *A*, dipeptidase; *B*, proteinase; *C*, papain.

Fig. 5. *A*, yeast dipeptidase (substrate leucylglycine) [Willstätter and Grassmann, 1926]; *B*, yeast aminopolypeptidase (substrate alanylglucylglycine) [Grassmann, 1927]; *C*, yeast proteinase (substrate gelatin) [Grassmann and Dyckerhoff, 1928]; *D*, yeast proteinase (substrate fibrin) [Grassmann and Dyckerhoff, 1928]; *E*, papain (substrate gelatin) [Willstätter and Grassmann, 1924].

Table IX.

p_H	5.2	6.0	7.0	8.0	9.0
Inactivation in control (%)	0	11	19	19	16
Inactivation in exp. assayed against control (%)	11	37	62	57	36

The procedure was as described above, the control and sample portions measuring 1.0 cc., and the period of heating being 30 minutes. Experiments were carried out at p_H 5.2, 5.8, 7.0, 7.5, 8.0 and 9.0.

All samples were assayed against the corresponding controls; 0.2 cc. of each was diluted to 10.0 cc. at p_H 7.5, and would thus have contained 0.25 unit per cc. The assay of the control samples against a dilution of the stock hormone solution showed that they had undergone little or no inactivation. The results are given in Table X and Fig. 4.

Table X.

p_H	5.2	5.8	7.0	7.5	8.0	9.0
Inactivation (%)	19	39	75	79	72	41

Oxytocic activity remaining after treatment of the hormone with the inactivating enzyme.

The enzyme used was prepared from English brewer's top yeast, and 200 mg. contained 10.2 units of dipeptidase, 2.8 units of aminopolypeptidase and no proteinase. A hormone solution (2.0 cc., containing 200 units per cc.) was adjusted to p_H 7.8 and mixed with a solution of the enzyme preparation (200 mg.)

in $M/3$ sodium phosphate buffer at p_H 7.8 (2.5 cc.) and water (2.5 cc.). The mixture was immediately diluted with water to 10.0 cc. A control sample (1.5 cc.) was removed as rapidly as possible into a 2.5 cc. flask which was immersed in a boiling water-bath. The procedure already described was followed, other samples being taken at appropriate intervals. The reaction remained constant at p_H 7.8 during the experiment, and after 24 hours about 40 % of the initial dipeptidase activity and 90 % of the aminopolypeptidase activity remained.

For assay the samples (2.0 cc.) were diluted to 5 cc. at p_H 7.5 and compared with a thousand-fold dilution (0.2 unit per cc.) of the stock hormone solution. Theoretically, therefore, the dilutions of the samples used for the assay would have contained 60 times as much active substance as the standard if no inactivation had occurred. The control solution was diluted a hundred-fold for assay, thus containing 0.3 unit per cc. The results are given in Table XI.

Table XI.

Time (hours)	0	0.25	0.5	1	24
Activity present (%)	23	1.0	0.7	0.7	0.7

This residual activity of 0.7 % was not due to histamine present as such in the stock hormone solution. In view of the method used to prepare this solution, it was unlikely that histamine should be found, but it was not impossible that traces might have been present as a result of adsorption on, or occlusion by, precipitates during the preparation. The oxytocic value of histamine in terms of hormonal activity having been ascertained as approximately 1.25 international standard units per 0.1 mg., the effect was investigated of heating at p_H 11 for 1 hour under similar conditions (a) a hormone solution containing 400 units, and (b) a histamine solution containing that quantity of base which had an activity approximately equivalent to 4 units (*i.e.* 1 % of the initial activity of the hormone solution). This treatment is well known to inactivate the hormone. If the activity remaining after prolonged treatment of the hormone with the inactivating enzyme was due to histamine, the hormone and histamine solutions should both have undergone inactivation to about the same value. In actual fact, however, the activity of the hormone solution fell practically to zero—definitely less than 0.016 unit, or 0.4 % of the activity remaining after prolonged enzymic inactivation. The activity of the histamine solution, on the other hand, was lowered only to 2 units, or 50 % of its initial value.

SUMMARY.

1. The hormone is inactivated by preparations of yeast dipeptidase, aminopolypeptidase and proteinase.
2. None of these enzymes is responsible for the inactivation, which is caused by a fourth enzyme present in these preparations. This has a p_H -optimum at p_H 7.4.
3. Papain preparations also contain the same enzyme.
4. The enzyme does not inactivate the hormone completely. There remains an activity of 0.7 % of the initial value; this is not caused by histamine present in the stock hormone solution, but is believed to be due to a derivative of the hormone produced by the action of the enzyme.

Our thanks are due to Dr W. Grassmann of Munich for gifts of dipeptidase and aminopolypeptidase, and to Messrs Boots Pure Drug Company, Nottingham, who generously presented the posterior lobe powder used in this work.

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CLXVIII. THE FORMATION OF HYDROGEN PEROXIDE IN CATALYTIC DEHYDROGENATION.

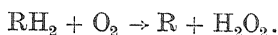
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(Received June 28th, 1933.)

ACCORDING to Wieland's dehydrogenation theory [1922, 1, 2; 1932] enzymic oxidation is effected by removal of hydrogen, the function of the enzyme being to activate certain hydrogen atoms of the substrate molecule and thus render them capable of removal by a hydrogen acceptor (oxygen, methylene blue, quinones and other substances). This theory is strongly supported by experimental evidence, perhaps the most important being the fact that the dehydrogenating enzymes exhibit marked specificity towards substrates but no specificity towards hydrogen acceptors. According to Wieland, catalytic dehydrogenation, such as that effected by the metals of the platinum group, is strictly analogous to enzymic dehydrogenation and is dependent on the activation of certain hydrogen atoms in the molecules by the catalysts and the subsequent removal of these hydrogen atoms by a hydrogen acceptor. It has been postulated that the hydrogen activations brought about by the dehydrases and the metallic catalysts are effected by the enzyme or catalyst forming a molecular complex with the substrate molecule resulting in the weakening of the bonds holding certain of the hydrogen atoms. The combination of hydrogen and oxygen, catalysed by the metals of the platinum group, is effected in a similar manner; a catalyst-hydrogen complex is first formed resulting in the activation of the hydrogen, followed by its subsequent removal from the complex by the oxygen.

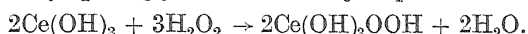
In aerobic dehydrogenations with both enzymes and catalysts the formation of hydrogen peroxide is demanded, the first step being the addition of the activated hydrogen atoms to the double bond of the oxygen molecule



In the aerobic dehydrogenations of xanthine and aldehydes in presence of their respective dehydrases it has been proved that hydrogen peroxide is formed [Wieland and Rosenfeld, 1929; Wieland and Macrae, 1930], and it is therefore probable that it is formed in all enzymic aerobic dehydrogenations; hydrogen peroxide is also an intermediate in the catalytic combination of hydrogen and oxygen [Hofmann, 1922]. The aim of this investigation was to establish that hydrogen peroxide is formed in catalytic dehydrogenation, as is demanded by the dehydrogenation theory. The catalytic dehydrogenation of alcohols to aldehydes [compare Wieland, 1912], which in itself is perhaps the most important dehydrogenation, has been investigated.

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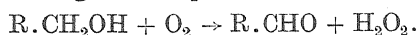
Difficulties in proving the intermediate formation of hydrogen peroxide in the aerobic dehydrogenation of alcohols were to be expected for the following reasons. Palladium black and the other catalysts of the platinum group decompose hydrogen peroxide very rapidly into water and oxygen [Wieland, 1921]. It is very probable that hydrogen peroxide might compete successfully with oxygen as hydrogen acceptor in the dehydrogenation of further quantities of alcohol. Finally hydrogen peroxide is capable of combining with aldehydes [Wieland and Winkler, 1923]. In recent years, however, cerous hydroxide has proved a valuable reagent for the detection of small quantities of hydrogen peroxide [Wieland and Rosenfeld, 1929; Wieland and Macrae, 1930; Macrae, 1931], and it seemed possible that it might be successfully employed in this investigation. When a suspension of cerous hydroxide is treated with hydrogen peroxide the suspension changes from white to a deep yellow-orange due to the formation of the very sparingly soluble ceric hydroperoxide



The ceric hydroperoxide may be centrifuged down and estimated iodimetrically; after some experience it is possible to estimate the amount of ceric hydroperoxide from the depth of the yellow coloration. Using cerous hydroxide $10^{-6} M$ or even less of hydrogen peroxide may be detected and $10^{-5} M$ may be determined with considerable accuracy.

Preliminary experiments on the catalytic combination of hydrogen and oxygen in presence of cerous hydroxide were carried out. Ceric hydroperoxide was formed and the observations of Hofmann [1922] have thus been verified. As was to be expected the ceric hydroperoxide formed represented only a very small percentage of that demanded by theory, the greater part of the hydrogen peroxide being decomposed or reduced to water before it could combine with the cerous hydroxide.

When aqueous solutions of ethyl alcohol and methyl alcohol containing cerous hydroxide in suspension were shaken with air in presence of a specially prepared palladium black, a small yield of ceric hydroperoxide was produced, thus demonstrating that hydrogen peroxide is formed in the aerobic catalytic dehydrogenation of these alcohols to the corresponding aldehydes. The reaction therefore proceeds according to the equation:



This provides substantiation of the Wieland dehydrogenation theory. The catalytic dehydrogenation of other compounds will be investigated.

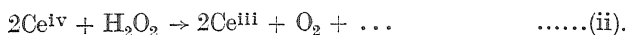
EXPERIMENTAL.

Preparation of the catalyst. Palladium black has been used throughout this investigation. The method of preparation employed was that of Willstätter and Waldschmidt-Leitz [1921]. It was found that immediately after preparation the catalyst was quite unsuitable for this work, being much too active. The palladium was therefore stored in a desiccator over potassium hydroxide and sulphuric acid for about 2 months; after that time the activity of the catalyst had fallen very considerably, but it still retained the power of catalysing the combination of hydrogen and oxygen and it also slowly effected the aerobic dehydrogenation of methyl alcohol and ethyl alcohol. Different preparations varied greatly in their suitability for this investigation.

Determination of ceric hydroperoxide. Ceric hydroperoxide may be estimated with considerable accuracy by colorimetric comparison with standard sus-

pensions, but confirmation by a titration method was thought desirable. The method described for the determination of ceric hydroperoxide by Wieland and Macrae [1930] requires catalase and therefore is not convenient. It was found, however, that this compound may be directly titrated iodimetrically, and although absolute accuracy is not obtained by this method, it was considered sufficiently accurate for these experiments.

When ceric hydroperoxide is decomposed by mineral acids it is converted into ceric salt and hydrogen peroxide (equation i). Iodimetric determination of the ceric salt and hydrogen peroxide is unfortunately impossible because a rapid interaction occurs between the ceric salt and the hydrogen peroxide resulting in the liberation of oxygen (equation ii).



When, however, the ceric hydroperoxide is decomposed by very dilute acetic acid in presence of potassium iodide, the liberation of oxygen is almost completely suppressed, and if the liberated iodine is immediately titrated with sodium thiosulphate fairly consistent results are obtained. Under these conditions it is only the ceric salt which liberates iodine; the liberation of iodine by hydrogen peroxide is very slow and may be ignored. 0.02 *M* cerous sulphate (2 cc.) was treated with 0.16 *M* sodium borate (2 cc.) and to the resulting suspension of cerous hydroxide 0.0079 *M* hydrogen peroxide (1 cc.) was added. After 10 minutes the yellow coloured mixture of cerous hydroxide and ceric hydroperoxide was centrifuged down, washed once with water and suspended in water (2 cc.) containing a few drops of *M* potassium iodide. *M* acetic acid (0.5 cc.) and two drops of a starch indicator solution were added, and the mixture was stirred. The suspension dissolved, and after 30 seconds the solution was titrated with 0.01*N* sodium thiosulphate. In 6 experiments the titrations were 0.52, 0.50, 0.58, 0.56, 0.48 and 0.52 cc. of 0.01*N* sodium thiosulphate—the theoretical value being 0.53 cc.

The catalytic combination of hydrogen and oxygen. 0.02 *M* cerous sulphate (2 cc.) was added to several aggregates of palladium black (about 50 mg.) in the vessel (100 cc.) of a shaking apparatus. After about 1 minute 0.16 *M* sodium borate (2 cc.) was added, which precipitated cerous hydroxide. The vessel was filled with a mixture of equal parts of hydrogen and air and was connected to a gasometer. The mixture was then shaken gently so that only a swirling motion was obtained, and the aggregates of palladium remained at the bottom of the vessel whilst the cerous hydroxide remained in suspension. There was a gradual absorption of gas, and after about 2 hours 15 cc. had been absorbed, and meantime there had been a gradual development of a yellow coloration due to the formation of ceric hydroperoxide. The cerous hydroxide-ceric hydroperoxide suspension was decanted from the palladium and estimated by the methods described above. This experiment was repeated several times (Table I). Other experiments were carried out using finely divided preparations of a more active catalyst. A more rapid absorption of gas was thus obtained, but no development of a yellow coloration was noted, partly because of the masking effect of the palladium black which formed a fine suspension. In these experiments only a very slight liberation of iodine resulted on centrifuging, washing and dissolving the cerous hydroxide in acetic acid in presence of potassium iodide; it is probable that the palladium effected almost complete decomposition of the hydrogen peroxide before it could combine with the cerous hydroxide.

Table I.

Palladium black mg.	0.02 M cerous sulphate cc.	0.16 M sodium borate cc.	Time hours	Gas absorbed cc.	Mols. of hydrogen peroxide estimated colori- metrically $\times 10^{-5}$	Mols. of hydrogen peroxide estimated iodi- metrically $\times 10^{-5}$
50 (feebly active)	2.0	2.0	2	21	0.45	0.43
50 "	2.0	2.0	2	16	0.45	0.37
50 "	2.0	2.0	1	10	0.30	0.28
50 "	2.0	2.0	2	15	0.45	0.49
50 (active)	2.0	2.0	0.5	30	—	<0.05
50 "	2.0	2.0	0.5	28	—	<0.05
—	2.0	2.0	4	0	0	0
—	2.0	2.0	4	0	0	0

Control experiments were carried out in the absence of palladium. Here there was no measurable absorption of gas and the cerous hydroxide suspension remained white; on subsequent treatment as above with acetic acid and potassium iodide it gave no liberation of iodine.

The amount of hydrogen peroxide obtained as ceric hydroperoxide represents only a very small percentage of the theory (about 1 %), but there is little doubt that the first step in the combination of hydrogen and oxygen results in the formation of hydrogen peroxide.

The catalytic dehydrogenation of methyl alcohol. 0.02 M cerous sulphate (2 cc.) was added to aggregates of palladium black (50 mg.) in the vessel of a shaking apparatus. After 1 minute 0.16 M sodium borate (2 cc.) was added and then pure methyl alcohol (0.5 cc.). As in the previous experiments the contents of the vessel were then gently shaken with air so that a swirling motion was obtained and the palladium remained at the bottom of the vessel whilst the cerous hydroxide remained in suspension. There was a slow absorption of gas and after several minutes the characteristic yellow colour of ceric hydroperoxide appeared and became more intense as the shaking continued. After about 5 hours 5 cc. of oxygen had been absorbed. The cerous hydroxide-ceric hydroperoxide

Table II.

Palladium black mg.	0.02 M cerous sulphate cc.	0.16 M sodium borate cc.	Methyl alcohol cc.	Time hours	Oxygen absorbed cc.	Mols. of hydrogen peroxide estimated colori- metrically $\times 10^{-5}$	Mols. of hydrogen peroxide estimated iodi- metrically $\times 10^{-5}$
50 (feebly active)	2.0	2.0	0.5	3	3.5	0.45	0.39
50 "	2.0	2.0	0.5	5	5.0	0.52	0.51
50 "	2.0	2.0	0.5	5	6.0	0.60	0.63
50 "	2.0	2.0	0.5	5	6.2	0.75	0.72
50 (active)	2.0	2.0	0.5	2	9.5	—	0.05
50 "	2.0	2.0	0.5	2	10.0	—	0.06
50 (feebly active)	2.0	2.0	—	5	0	0	0
50 "	2.0	2.0	—	5	0	0	0
—	2.0	2.0	0.5	5	0	0	0
—	2.0	2.0	0.5	5	0	0	0

suspension was decanted from the palladium and the characteristic odour of formaldehyde was perceptible. The ceric hydroperoxide was determined colorimetrically and iodimetrically as in the previous experiments. The experiment was repeated several times (Table II). Experiments using a more active finely divided catalyst were less successful. The following control experiments were carried out: cerous hydroxide and palladium black were shaken for 5 hours with

air; cerous hydroxide and methyl alcohol were shaken for 5 hours with air in the absence of palladium black. In both these experiments no oxygen was absorbed, no yellow coloration developed and no iodine was liberated in the iodimetric estimation.

The yield of ceric hydroperoxide represents about 1 % of the theory.

The catalytic dehydrogenation of ethyl alcohol. The procedure was the same as in the experiments with methyl alcohol. Pure ethyl alcohol was used and a slow absorption of oxygen accompanied by the development of the characteristic yellow colour of ceric hydroperoxide resulted. At the end of the experiment the

Table III.

Palladium black mg.	0.02 M cerous sulphate cc.	0.16 M sodium borate cc.	Ethyl alcohol cc.	Time hours	Oxygen absorbed cc.	Mols. of hydrogen peroxide estimated colori- metrically $\times 10^{-5}$	Mols. of hydrogen peroxide estimated iodi- metrically $\times 10^{-5}$
50 (feebly active)	2.0	2.0	0.5	5	5.8	0.67	0.66
50 "	2.0	2.0	0.5	4	5.0	0.60	0.53
50 "	2.0	2.0	0.5	4	4.8	0.60	0.57
50 "	2.0	2.0	0.5	3	4.0	0.45	0.46
50 (active)	2.0	2.0	0.5	2	11.0	—	0.04
50 "	2.0	2.0	0.5	1.5	8.5	—	0.06
—	2.0	2.0	0.5	5	0	0	0
—	2.0	2.0	0.5	5	0	0	0

odour of acetaldehyde was perceptible. The ceric hydroperoxide was estimated as in the experiments with methyl alcohol (Table III). As before an active finely divided catalyst was less suitable. A control experiment without palladium gave no gas absorption, no yellow coloration and no iodine liberation in the iodimetric determination.

SUMMARY.

1. It has been verified that hydrogen peroxide is formed intermediately in the catalytic combination of hydrogen and oxygen.
2. Hydrogen peroxide has been detected in the catalytic aerobic dehydrogenation of methyl alcohol and ethyl alcohol. The Wieland theory of dehydrogenation is thus substantiated.

I am indebted to Dr J. M. Gulland for advice.

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CLXIX. THE INFLUENCE OF VARIATIONS IN SYSTEMIC ACID-BASE BALANCE UPON CARBOHYDRATE TOLERANCE IN NORMAL SUBJECTS.

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(Received June 29th, 1933.)

THE relationship of changes in acid-base balance of the blood to carbohydrate storage has attracted the interest of several workers. Elias [1913], working with dogs, showed that the administration of HCl by mouth was followed by hyperglycaemia and glycosuria. Underhill [1916] showed that administration of acid tended to raise the blood-sugar, whereas alkalis reduced it. Hendrix and McAmis [1924] found that the alkalosis produced in dogs by the injection of hydrazine sulphate was associated with hypoglycaemia. Peters and Geyelin [1917], studying adrenaline hyperglycaemia in three human diabetics and two normal subjects, suggested that the hyperglycaemia and glycosuria were the results of an intermediate acidosis. Haldane [1924] reports "an upset of carbohydrate metabolism like that of diabetes" during the alkalosis induced by over-breathing or alkali ingestion. This observer also states that during ammonium chloride acidosis the ingestion of 100 g. of glucose produced hyperglycaemia and glycosuria.

The subject is one of considerable interest owing to the association of severe acidosis with human diabetes. It seems possible that the acidosis of diabetes, which is incidentally the severest acidosis met with in human beings except for some cases moribund from uraemia, may have a contributory effect upon the changes in carbohydrate metabolism encountered in this disease. Unfortunately most of the experimental work up to the present suffers from the obvious drawback of having been performed on laboratory animals and not on the human subject.

The object of the present investigation was to study carbohydrate tolerance in young healthy male adults during experimental acidosis of a degree comparable to that encountered in severe untreated diabetes. Such an acidosis can readily be induced by the oral ingestion of massive doses of ammonium chloride [Haldane, 1921]. Similar observations were performed on the same subjects during alkalosis induced by the ingestion of large amounts of sodium bicarbonate.

Method. Diet was kept constant throughout, except as regards water and salts. Metabolic changes occurring during acidosis and alkalosis were followed by estimations of the CO₂-combining power of the blood-plasma and of the total daily excretion of acid, chloride and ammonia in the urine. In each case there was a preliminary control period on normal diet, followed by a period of

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Table I. *Urine analyses.*

Day	Volume cc.	Total daily output			CO ₂ plasma vols. %	Ammonium chloride or sodium bicarbonate ingested	Remarks
		Acid cc.	Ammonia cc.	Chlorides			
		N/10 NaOH	N/10 NaOH	g. NaCl			
Subject 1:							
1	1325	239	599	11.6	—	Nil	—
2	1530	201	563	8.75	—	Nil	—
3	1220	220	488	4.9	63	Nil	Control glucose tolerance test
4	1740	400	974	16.2	—	15 g. NH ₄ Cl	Symptoms: headache, insomnia; continuous nausea, no actual vomiting. No dyspnoea. Only 17 g. on account of nausea. Better next day
5	2000	520	1625	27.0	—	20 g. NH ₄ Cl	
6	1960	643	2399	24.5	—	17 g. NH ₄ Cl	
7	1000	376	1972	9.3	33	Nil	Acidosis glucose tolerance test
8	945	208	431	6.9	—	Nil	—
9	760	207	403	4.1	—	Nil	—
10	1090	76	283	5.7	—	20 g. NaHCO ₃	Symptoms: marked thirst, otherwise nil
11	2030	41	81	2.4	—	40 g. NaHCO ₃	
12	2560	Nil	51	3.9	—	70 g. NaHCO ₃	
13	—	—	—	—	68	—	Alkalosis glucose tolerance test
Subject 2:							
1	—	—	—	—	52	Nil	Control glucose tolerance test
2	1020	379	388	9.4	—	Nil	—
3	1130	289	378	13.6	—	Nil	—
4	1025	291	394	10.5	—	Nil	—
5	2320	483	724	18.7	—	15 g. NH ₄ Cl	Salivation; slight nausea
6	2420	600	1142	29.6	—	20 g. NH ₄ Cl	Nausea more marked; watery diarrhoea
7	2030	665	1697	26.1	—	20 g. NH ₄ Cl	Headache; severe nausea; vomited at 3 a.m.
8	—	—	—	—	32	Nil	Acidosis glucose tolerance test
9	870	390	313	9.2	—	Nil	—
10	1390	234	289	14.2	—	Nil	—
11	1680	275	262	14.4	—	Nil	—
12	2050	156	172	10.9	—	20 g. NaHCO ₃	—
13	2510	80	90	14.4	—	20 g. NaHCO ₃	Symptoms nil
14	2690	81	83	14.8	—	20 g. NaHCO ₃	—
15	—	—	—	—	57	Nil	Alkalosis glucose tolerance test
Subject 3:							
1	—	—	—	—	66	Nil	Control glucose tolerance test
2	1130	429	760	15	—	Nil	—
3	1205	414	733	14	—	Nil	—
4	1330	463	734	16	—	20 g. NH ₄ Cl	Symptoms nil except for increased salivation
5	2080	649	1440	30	—	20 g. NH ₄ Cl	
6	2490	936	1972	36	—	20 g. NH ₄ Cl	
7	2170	1068	2930	35	—	20 g. NH ₄ Cl	
8	2470	929	3359	39	38	25 g. NH ₄ Cl	Acidosis glucose tolerance test
9	1385	526	1233	7	—	Nil	—
10	1600	307	678	8	—	20 g. NaHCO ₃	Symptoms nil
11	2310	129	286	13	—	25 g. NaHCO ₃	Symptoms nil
12	2360	Nil	Nil	9	—	30 g. NaHCO ₃	Symptoms: marked tingling in hands and feet
13	—	—	—	—	68	—	Alkalosis glucose tolerance test

acidosis and finally a period of alkalosis, diet being kept constant throughout. Glucose tolerance was estimated during each period by following the changes in venous blood-sugar after the oral ingestion of 100 g. of B.D.H., A.R. standard dextrose dissolved in 250 cc. of water. Specimens of blood were withdrawn at 15-minute intervals during the first hour of the test, and then at 30-minute intervals. Urine specimens were examined for reducing substances at half-hour intervals.

Urine analysis. A. Quantitative.

- (1) Total acidity by Folin's method.
- (2) Ammonia by Malfatti's formaldehyde titration method. This includes amino-acids.
- (3) Chlorides by Volhard's method.

B. Qualitative.

- (1) Protein by salicylsulphonic acid.
- (2) Sugar by Benedict's qualitative method.
- (3) Ketone bodies by Rothera's method.

Blood-sugar was determined in specimens of venous blood by Folin and Wu's colorimetric method. The figures obtained include non-glucose reducing substances [Herbert and Groen, 1929].

Induction of acidosis. This was accomplished by the ingestion of ammonium chloride in 2 % solution. The total amounts taken were 52 g., 55 g. and 105 g. in 3 days.

Induction of alkalosis. Sodium bicarbonate suspended in water was ingested in amounts as shown in Table I. The mixture was stirred up and immediately swallowed. Thus in spite of low solubility 5 or 10 g. could be taken in a small volume of water.

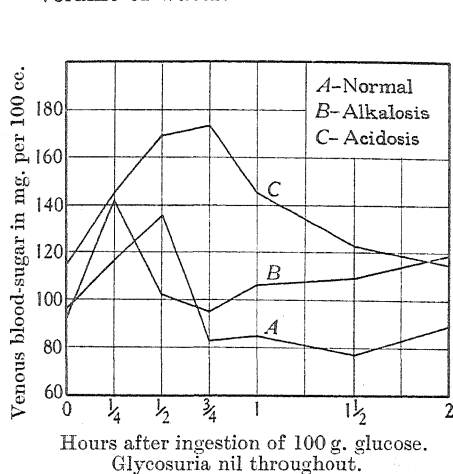


Fig. 1. Blood-sugar curves in subject 1.

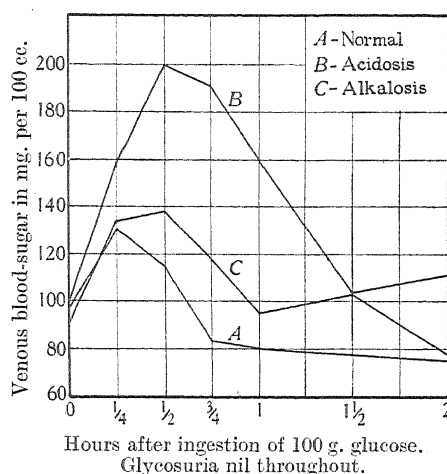


Fig. 2. Blood-sugar curves in subject 2.

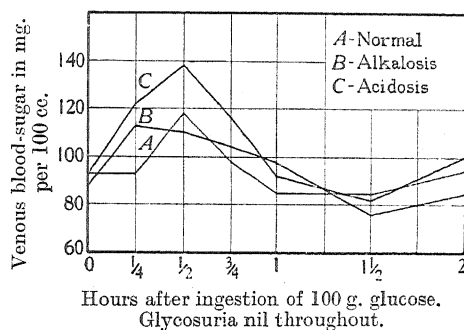


Fig. 3. Blood-sugar curves in subject 3.

Determination of CO₂-combining power of plasma. In each case this determination was performed on the first specimen of blood (fasting) taken for the glucose tolerance test. About 5 cc. of venous blood were rapidly withdrawn

under oil without stasis, oxalated and rapidly centrifuged under oil. The plasma was sucked off and equilibrated with the subject's own alveolar air. The CO_2 content of 1 cc. of equilibrated plasma was then determined in the Van Slyke apparatus, duplicate determinations being performed in each case. Results, after appropriate corrections for temperature and pressure, are shown in Table I.

DISCUSSION AND CONCLUSIONS.

It was found possible by the ingestion of large amounts of ammonium chloride to reduce the alkali reserve of the blood-plasma to a level comparable with that found in diabetic coma and to raise the urinary excretion of acid by 100–200 % and of ammonia by 300 %. The acidosis so produced caused a definite failure in glucose tolerance, shown by hyperglycaemia and slow return of the blood-sugar to fasting level following glucose ingestion, but without any trace of glycosuria. These points are strikingly shown in subjects 1 and 2, but are less marked though still apparent in subject 3 for whom the curves for venous blood tend to be of a flat type. The absence of glycosuria is surprising in view of the fact that in two of the subjects the blood-sugar level exceeded what is usually regarded as the normal renal threshold.

The ingestion of sodium bicarbonate in large doses, sufficient to reduce the urinary excretion of ammonia and acid to zero, was not followed by marked increase in the CO_2 -combining power of the plasma. In subject 1 the last dose of alkali (10 g.) was taken $2\frac{1}{2}$ hours before the determination of plasma- CO_2 . These observations were carefully performed on three occasions with the same result. Palmer and Van Slyke [1917] found it necessary to raise the plasma-bicarbonate to 75–80 volumes % by alkali ingestion before the sum of urinary ammonia and titratable acid fell to zero. It appears, however, that the CO_2 -combining power of the plasma may sometimes be a misleading guide to the extent of the total upset in acid-base balance following alkali ingestion.

Ingestion of alkali caused very little variation in the blood-sugar response to glucose ingestion. There was a tendency to a sharper rise in the initial phase, but thereafter the curves were little different from normal.

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CLXX. STUDIES ON THE RELATIONSHIP BETWEEN CHEMICAL CONSTITUTION AND PHYSIOLOGICAL ACTION.

V. MOLECULAR DISSYMMETRY AND PHYSIOLOGICAL ACTIVITY.

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(Received June 30th, 1933.)

WHEN a molecularly dissymmetric substance possesses pharmacological activity it is frequently, although not invariably, found that one optical isomeride is considerably more potent than the other. The work carried out on this subject has been reviewed by Cushny [1926], who himself made a detailed pharmacological comparison of the antimeric adrenalines and hyoscyamines. Largely as a result of his studies on these substances, Cushny regarded optical activity as a factor which is quite distinct from general structure in determining the magnitude of the specific pharmacological activity of a molecule, and this view is, we believe, the one which is currently held.

Cushny regarded the difference between the physiological activities of two optical isomerides as analogous to the different behaviours of the antimeric forms towards optically active acids and was of the opinion that an explanation of the influence of molecular dissymmetry on physiological activity would be found along the lines of this analogy, possibly, for example, by the more active isomeride forming a compound with an optically-active specific receptor in the tissues which possessed different physical properties from that formed with its enantiomorph.

It is the purpose of the present communication to elaborate an alternative view according to which there appears to be no reason for differentiating between molecular dissymmetry and structure in regard to the manner in which they influence physiological activity. On the contrary, it is considered that the different physiological activities of optical isomerides may frequently be ascribed to circumstances which are identical with those which cause different symmetrical molecules to exhibit different physiological activities; in other words, that molecular dissymmetry is, of itself, without influence on physiological activity, and that both it and the optical activity with which it is associated are accidental accompaniments, in optical isomerides, of different molecular arrangements which differ in their ability to cause the development of a particular physiological effect for precisely the same reasons that two structural isomerides so differ.

Before considering any definite examples, the theoretical basis of our suggestion must be explained. This starts from the postulate that a drug is attached to its specific receptor in the tissues in such a manner that a considerable proportion of the drug molecule is involved. If an asymmetric carbon atom is

present, three of the groups linked to this atom may be concerned in the process. The exact nature of the attachment is immaterial to the main argument, but it is our view that this may be effected either by normal valencies or by adsorptive or other forces; or it may be of a loose type, somewhat analogous to the attachment of a glove to the hand, involving the contour of a large part of the molecule rather than points on its surface; or, finally, a combination of these possibilities may be involved. Whatever the mechanism, the position of affairs may be represented by the diagrams in Fig. 1. Of these, III and IV represent, in the conventional manner, two enantiomorphs, while VI depicts diagrammatically the surface of the specific receptor in the tissues. For the drug molecule to produce a maximum physiological effect it must, according to the above postulate, become attached to the receptor in such a manner that the groups *B*, *C* and *D* in the drug coincide respectively with *B'*, *C'* and *D'* in the receptor. Such coincidence can only occur with one of the enantiomorphs (III), and this consequently represents the more active form of the drug. Let it now be supposed that the dissymmetry of III is abolished by replacing the group *A* by a second group *B*. The resultant molecule, represented by V, retains unchanged that part of the structure of III, *i.e.* the base *BCD* of the tetrahedron, which is concerned with its attachment to the specific receptor and must therefore be considered capable, despite the absence of molecular dissymmetry, of exerting its physiological activity with an intensity numerically equal to that of III, except in so far as this activity is changed by modifications in such of its properties as are not directly concerned with its attachment to the receptor. It will be noted that V possesses a second face, shaded in the diagram, containing the groups *BCD*. This face, however, corresponds with the base of IV and, like this, cannot be brought into coincidence with the receptor.

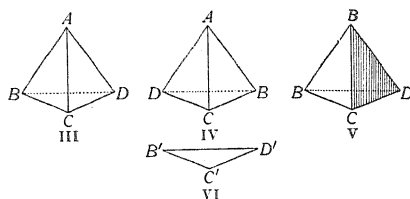
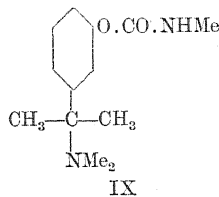
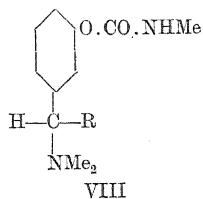
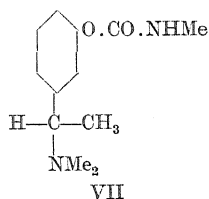


Fig. 1.

In order to apply this generalised argument to a particular drug it must be decided which of the four groups linked to the asymmetric carbon atom are concerned in the attachment of the drug molecule to the receptor. Such a decision can be reached with a fair degree of certainty for *l*-adrenaline from a consideration of the abundant evidence which is available relating structure to sympathomimetic action, and there seems little doubt that the groups in question are the basic, the aromatic, and the alcoholic hydroxyl groups. Our reasons for this conclusion, as well as our views regarding the mechanism of attachment, are briefly as follows. (1) The basic group is a feature common to all the sympathomimetic bases. From its nature it appears probable that this group is linked to the receptor by means of normal valencies. (2) The aromatic group is also necessary for the development of any appreciable activity. Whether or no this is attached by normal valencies is more problematical. We think, however, that it should be regarded as a possibility that the group, as a whole, "fits" the receptor, but that when it carries phenolic hydroxyl groups in the *m*- or *p*- positions it becomes more firmly attached by means of normal valencies. (3) The alcoholic hydroxyl group is necessarily concerned in the attachment, since it is the presence of this group which is responsible for the outstanding activity of *l*-adrenaline. It is nevertheless difficult to say whether it is combined chemically with the receptor or whether it merely causes a better "fit" of the drug to the receptor.

Of the four groups linked to the asymmetric carbon atom in *l*-adrenaline it is thus the hydrogen atom, represented by *A* in III, which plays no part in the attachment of the drug to the receptor. If this group were replaced by a hydroxyl group a symmetrical compound corresponding with V would result which, according to the above argument, would possess a physiological activity equal to that of *l*-adrenaline except in so far as this activity was modified by changes in the physical properties of the compound due to the presence of the second hydroxyl group. Unfortunately such a compound is not capable of existence and cannot, therefore, be utilised to test our hypothesis. A similar test, scarcely less satisfactory, can however be made. If IV be regarded as representing *d*-adrenaline, it is clear that the hydroxyl group (*B*) cannot be concerned with the attachment of the drug to its receptor, but that this must be brought about by the face *ACD* involving the hydrogen atom (*A*), and the smaller physiological activity of this isomeride is attributed to the less perfect combination which results. By substituting a hydrogen atom for the hydroxyl group in *d*-adrenaline a symmetrical molecule would be obtained which, on the hypothesis which we have advanced, should combine as readily as *d*-adrenaline with the receptor and should therefore exhibit, with the above reservation regarding physical properties, the same physiological activity as this compound. The symmetrical compound in question is 3:4-dihydroxy- β -phenylethylmethylamine. While the activity of this compound has not been compared directly with that of *d*-adrenaline, data are available from which the relative activities of the two bases can be calculated. Thus, according to Barger and Dale [1910], the pressor activity of *dl*-adrenaline is seven times as great as that of 3:4-dihydroxy- β -phenylethylmethylamine, while Cushny's results show that *l*-adrenaline is 12-15 times as active as *d*-adrenaline. It follows that *d*-adrenaline is 1.07 to 0.87 times as active as 3:4-dihydroxy- β -phenylethylmethylamine, a result which corresponds with that deduced from our hypothesis much more closely than might have been anticipated.

One such favourable result cannot, of course, be regarded as establishing the correctness of our suggestion, and we have therefore attempted to obtain further experimental support for it with another group of drugs, namely the synthetic miotics which have formed the subject of the preceding papers of this series (for references, see Stedman [1931]). Among these drugs, miotine (VII)



possesses exceptional miotic activity, and one which is considerably greater than that of the methylurethane of *m*-hydroxybenzyltrimethylamine (VIII; R = H). Its large activity is thus associated with the introduction of the methyl group into the side-chain, which simultaneously creates an asymmetric carbon atom in the molecule. We were inclined originally to ascribe the high physiological activity of this substance to the latter circumstance, but the view outlined above now offers an alternative explanation. According to this view miotine, and the miotics of this class in general, must become attached to their receptor by means of the basic and $\text{NH}(\text{CH}_3)_2 \cdot \text{CO} \cdot \text{O} \cdot \text{C}_6\text{H}_4$ -groups, since these two groups

are together responsible for the physiological properties of these compounds. Since the presence of the methyl group in miotine is associated with high physiological activity it must further be assumed that, in the more active isomeride, this group causes a more perfect combination to take place between drug and receptor than would occur in its absence. In the less active isomeride, on the other hand, the methyl group must be directed away from the receptor and consequently cannot directly influence the fixation of the drug. It is thus clear that a compound of the formula IX, in which the molecular dissymmetry of miotine has been abolished by the introduction of a second methyl group, should possess a physiological activity equal to that of the more active form of miotine except in so far as this activity is modified by changes in the physical properties of the compound. Similarly, the less active form of miotine should possess an activity equal to that of VIII ($R = H$).

In order to test these deductions, certain of the properties of the compounds VII, VIII ($R = H$) and IX have been compared. In addition some urethanes of the general formula VIII, in which $R = Et, Pr^a$, and Ph , have been similarly examined in order to obtain data from which the influence of increased hydrocarbon content on the physiological activity of this type of substance might to some extent be judged. Measurements have also been made with the corresponding methiodides. The latter, however, clearly contribute little to the present discussion, since the methiodide from miotine does not show any markedly greater activity than that of its parent substance (VIII; $R = H$). A description of the various compounds examined will be published elsewhere.

RESULTS.

Miotic activities. The above urethanes were first compared with respect to their action on the pupil. For this purpose, one drop of a solution of the hydrochloride or methiodide in physiological saline was instilled by one of us (E. S.) into the eye of a cat. To ensure that the drops were, as far as possible, of the same size, the same pipette was used throughout the experiments. Individual variations in different animals were avoided by carrying out all the tests on one animal. Alternate eyes were used, but on no occasion was an experiment carried out until both eyes were completely normal. This usually necessitated an interval of 24, and in some cases 48, hours between successive experiments. The results are tabulated below. Slight salivation usually occurred shortly after the instillation of the drug; with Nos. 6 and 12, however, salivation was considerable. Racemic miotine was necessarily used for these experiments, since,

Methylurethane	Conc. %	Miosis
1. $m\text{-NHMe.CO}_2\text{.C}_6\text{H}_4\text{.CH}_2\text{.NMe}_2\text{.HCl}$	0.1	Slight but distinct after 20-25 mins.
2. $m\text{-NHMe.CO}_2\text{.C}_6\text{H}_4\text{.CHMe.NMe}_2\text{.HCl}$	0.1	Very marked; commenced in 15 mins. and reached maximum in 25 mins.
3. $m\text{-NHMe.CO}_2\text{.C}_6\text{H}_4\text{.CMe}_2\text{.NMe}_2\text{.HCl}$	0.1	Similar to 2 but effect somewhat greater
4. $m\text{-NHMe.CO}_2\text{.C}_6\text{H}_4\text{.CHEt.NMe}_2\text{.HCl}$	0.1	Similar to 1 but effect greater
5. $m\text{-NHMe.CO}_2\text{.C}_6\text{H}_4\text{.CHPr}^a\text{.NMe}_2\text{.HCl}$	0.1	Similar to 1 and 4
6. $m\text{-NHMe.CO}_2\text{.C}_6\text{H}_4\text{.CHPh.NMe}_2\text{.HCl}$	0.1	Nil
7. $o\text{-NHMe.CO}_2\text{.C}_6\text{H}_4\text{.CH}_2\text{.NMe}_2\text{.HCl}$	1.0 0.1	Slight after 30-40 mins. Commenced in 15 mins. Probably greater than 1
8. $p\text{-NHMe.CO}_2\text{.C}_6\text{H}_4\text{.CH}_2\text{.NMe}_2\text{.HCl}$	0.1	Nil
9. $m\text{-NHMe.CO}_2\text{.C}_6\text{H}_4\text{.CMe}_2\text{.NMe}_2\text{.I}$	1.0	Distinct after 15-20 mins.; maximum in 25-30 mins.
10. $m\text{-NHMe.CO}_2\text{.C}_6\text{H}_4\text{.CHEt.NMe}_2\text{.I}$	1.0	Very slight after 30 mins. Less than 9
11. $m\text{-NHMe.CO}_2\text{.C}_6\text{H}_4\text{.CHPr}^a\text{.NMe}_2\text{.I}$	1.0	Nil
12. $m\text{-NHMe.CO}_2\text{.C}_6\text{H}_4\text{.CHPh.NMe}_2\text{.I}$	1.0	Slight after 30-40 mins.

at the time they were carried out, the antimeric forms were not available. This, however, does not invalidate the results. According to our argument a compound of formula IX should possess an activity equal to that of the more active form of miotine and hence greater than that of *dl*-miotine. This involves, it is true, the assumption that the two forms of miotine differ in activity, but this assumption, as will be seen from a later section, has now been verified.

The above results are clearly in accordance with our hypothesis. The general effect of increasing the hydrocarbon content of the molecule is to diminish its miotic activity. This is exemplified by the activities of 4 and 5, which are much smaller than that of miotine (2). On the other hand, the introduction of the second methyl group into miotine with the production of 3, although this necessarily increases the hydrocarbon content of the molecule, is associated with an increase in miotic activity.

The miotic activities of the methylurethanes of the isomeric hydroxybenzyl-dimethylamines (1, 7 and 8) have been re-examined since, according to Stedman [1929], they are in the order $o > p > m$ while White and Stedman [1931] state that the *m*-compound is more active than the *p*. In agreement with the latter result the order is now found to be $o \approx m > p$.

Inhibitory activities towards liver esterase. It has been shown by Stedman and Stedman [1931; 1932] that, in addition to possessing characteristic pharmacological properties, urethanes of the type under consideration also inhibit the activity of liver esterase. Since the suggestion which we have made regarding the influence of molecular dissymmetry on physiological activity applies equally well to the influence of the same factor, either in substrate or inhibitor, on enzymic activity, we have compared the inhibitory activities of the above urethanes on the liver esterases from the cat and the pig. As in the miotic experiments, *dl*-miotine was employed.

The enzyme preparations were made by extracting the desiccated liver powder with dilute ammonia, precipitating some impurities from this extract with acetic acid, and then dialysing the centrifugate, according to the detailed procedure previously described. A method, based upon that of Knaff-Lenz [1923], has however now been employed for following the hydrolysis of the substrate. To a solution of 0.25 cc. of methyl butyrate in 100 cc. of water warmed to 30° were added 10 drops of a solution of bromothymol blue and sufficient NaOH to bring the solution to about p_H 7.6 as shown by the indicator. A suitable volume of the enzyme or enzyme-inhibitor solution was then added and the p_H of the solution maintained as constant as possible at the above value by the addition, drop by drop as required, of 0.02 *N* NaOH. Burette readings were taken at regular intervals. The inhibitor was left overnight in contact with the enzyme.

The results obtained with liver esterase from the cat are given in Table I. Since all the inhibitors examined were of the general formula



they are described in this and the following tables by the groups which are represented by RR' in this formula. It should be mentioned that considerable difficulty was experienced in obtaining consistent results with the enzyme from the cat and many measurements had to be discarded as worthless. The cause of this inconsistency was ultimately traced to the fact that the acidity of an extract of liver esterase from this animal slowly increases on standing. Now, the inhibitory activities of the urethanes in question vary with the p_H of the solution in which they are in contact with the enzyme. Since the measurements with

Table I.

Enzyme: liver esterase from cat. Substrate: methyl butyrate. p_H 7.6. $t=30^\circ$.
 Final conc. of inhibitors: 4×10^{-7} molar.

Inhibitor	Alkali in 5 min. periods	Total	Percentage inhibition
Hydrochlorides			
Control	4.2, 4.2, 4.1, 4.2	16.6	—
H, H	1.2, 1.2, 1.1, 1.2	4.7	72
H, Me	1.2, 1.3, 1.2, 1.3	5.0	70
Me, Me	1.0, 0.9, 1.0, 1.0	3.9	77
H, Et	1.3, 1.3, 1.3, 1.3	5.3	68
H, Pr ^a	1.2, 1.2, 1.4, 1.3	5.1	69
H, Ph	1.7, 1.5, 1.8, 1.6	6.6	60
Hydrochlorides			
Control	4.6, 5.0, 4.8, 4.7	19.1	—
H, H	1.1, 1.3, 1.3, 1.3	5.0	74
H, Me	1.0, 1.2, 1.1, 1.1	4.4	77
Me, Me	0.9, 0.9, 1.1, 1.0	3.9	80
H, Et	1.2, 1.2, 1.3, 1.3	5.0	74

the individual drugs were usually made seriatim, they were carried out with a solution which was becoming progressively more acid and hence were not comparable. In the experiments recorded in Table I, this difficulty was overcome by carrying out each group of experiments simultaneously, the enzyme solution having been brought to p_H about 7.6 by the addition of alkali immediately prior to mixing it with the inhibitors. A buffer could hardly be employed, since the amount necessary to prevent the change in acidity would have interfered with the accuracy of the subsequent titration. We are unable definitely to explain the cause of this liberation of acid. It did not appear to be due to the action of micro-organisms since the enzyme extract was saturated with chloroform in order to keep it sterile. The most probable explanation is that it was caused by the presence of another enzyme, possibly an oxidase, in the extract.

An inspection of Table I shows that the results run parallel with those obtained in the miosis experiments. Increase in the size of the alkyl group diminished the inhibitory activity of the urethane, while the introduction of the second methyl group into miotine increased it.

Tables II and III give the results obtained with liver esterase from the pig. The enzyme preparation used in the first series was made from a liver powder which was over a year old; that employed in the second was from a freshly prepared powder. This apparent duplication of experimental material was made because the activity towards methyl butyrate of an extract from an old powder is always considerably less than that from a new one, whereas the reverse holds when the substrate is tributyrin, and it was desired to ascertain if this diminution in activity was accompanied by any change in the inhibition phenomena. It can be seen from the tables that this is not the case. In these experiments the enzyme was left overnight in contact with the inhibitor in the presence of a small amount of buffer (p_H 8.9).

The results obtained with the enzyme from the pig do not at first sight appear to be so favourable to our hypothesis as do those described above. While they are satisfactory to the extent that the symmetrical compound (Me, Me) possesses a greater inhibitory activity than its isomeride (H, Et), the former is nevertheless considerably less active than miotine. When, however, it is recalled

Table II.

Enzyme: liver esterase from pig. Substrate: methyl butyrate. p_H 7.6. $t=30^\circ$.
 Final conc. of inhibitors: hydrochlorides, 2.5×10^{-7} ; methiodides, 2.5×10^{-6} molar.

Inhibitor	Alkali in 5 min. periods	Total	Percentage inhibition
Hydrochlorides			
Control	1.8, 1.9, 2.0, 1.9	7.6	—
H, H	1.4, 1.4, 1.35, 1.45	5.6	26
H, Me	1.25, 1.15, 1.25, 1.3	4.95	35
Me, Me	1.3, 1.4, 1.4, 1.35	5.45	28
H, Et	1.4, 1.5, 1.45, 1.4	5.75	24
H, Pr ^a	1.4, 1.45, 1.55, 1.6	6.0	21
H, Ph	1.1, 1.1, 1.2, 1.1	4.5	41
Methiodides			
Control	2.0, 2.05, 1.95, 2.0	8.0	—
H, H	0.9, 1.0, 1.0, 0.95	3.85	52
H, Me	1.05, 0.95, 0.9, 1.0	3.9	51
Me, Me	0.9, 0.95, 0.9, 0.95	3.7	54
H, Et	1.05, 1.0, 1.05, 0.95	4.05	49
H, Pr ^a	1.05, 1.05, 1.05, 1.0	4.15	48
H, Ph	1.25, 1.2, 1.1, 1.15	4.7	41

Table III.

Enzyme: liver esterase from pig. Substrate: methyl butyrate. p_H 7.6. $t=30^\circ$.
 Final conc. of inhibitors: hydrochlorides, 2.5×10^{-7} ; methiodides, 5×10^{-7} molar.

Inhibitor	Alkali in 5 min. periods	Total	Percentage inhibition
Hydrochlorides			
Control	5.4, 5.5, 5.6, 5.5	22.0	—
H, H	3.3, 3.4, 3.4, 3.4	13.5	39
H, Me	2.7, 2.6, 2.8, 2.7	10.8	51
Me, Me	3.1, 3.3, 3.4, 3.3	13.1	40
H, Et	3.5, 3.4, 3.5, 3.5	13.9	37
H, Pr ^a	3.8, 3.9, 3.9, 3.9	15.5	30
H, Ph	3.2, 3.0, 3.1, 3.0	12.3	44
Methiodides			
Control	5.2, 5.4, 5.4, 5.4	21.4	—
H, H	3.6, 3.5, 3.6, 3.5	14.2	34
H, Me	3.6, 3.6, 3.5, 3.6	14.3	33
Me, Me	3.7, 3.7, 3.8, 3.7	14.9	30
H, Et	3.7, 3.7, 3.6, 3.6	14.6	32
H, Pr ^a	3.7, 3.6, 3.7, 3.7	14.7	31
H, Ph	3.5, 3.5, 3.5, 3.6	14.1	34

that the general effect of increasing the hydrocarbon content is to diminish the activity of the compound, it is clear that if the difference between the inhibitory activities of *d*- and *l*-miotine is, in this case, small the hydrocarbon effect might outweigh that due to configuration. Since the above experiments were carried out the preparation of the antimeric miotines by Macdonald and Stedman [1932] has enabled us to test this point, and it has, in fact, been found that there is little difference between the inhibitory activities of the two forms towards liver esterase from the pig, although the *l*-compound is slightly the more active. The results, therefore, can be regarded as consistent with our theory.

Action on intestine. A detailed pharmacological comparison of *d*- and *l*-miotine, which have now become available, is being made by Dr A. C. White, who has informed us that, in those experiments which he has so far carried out,

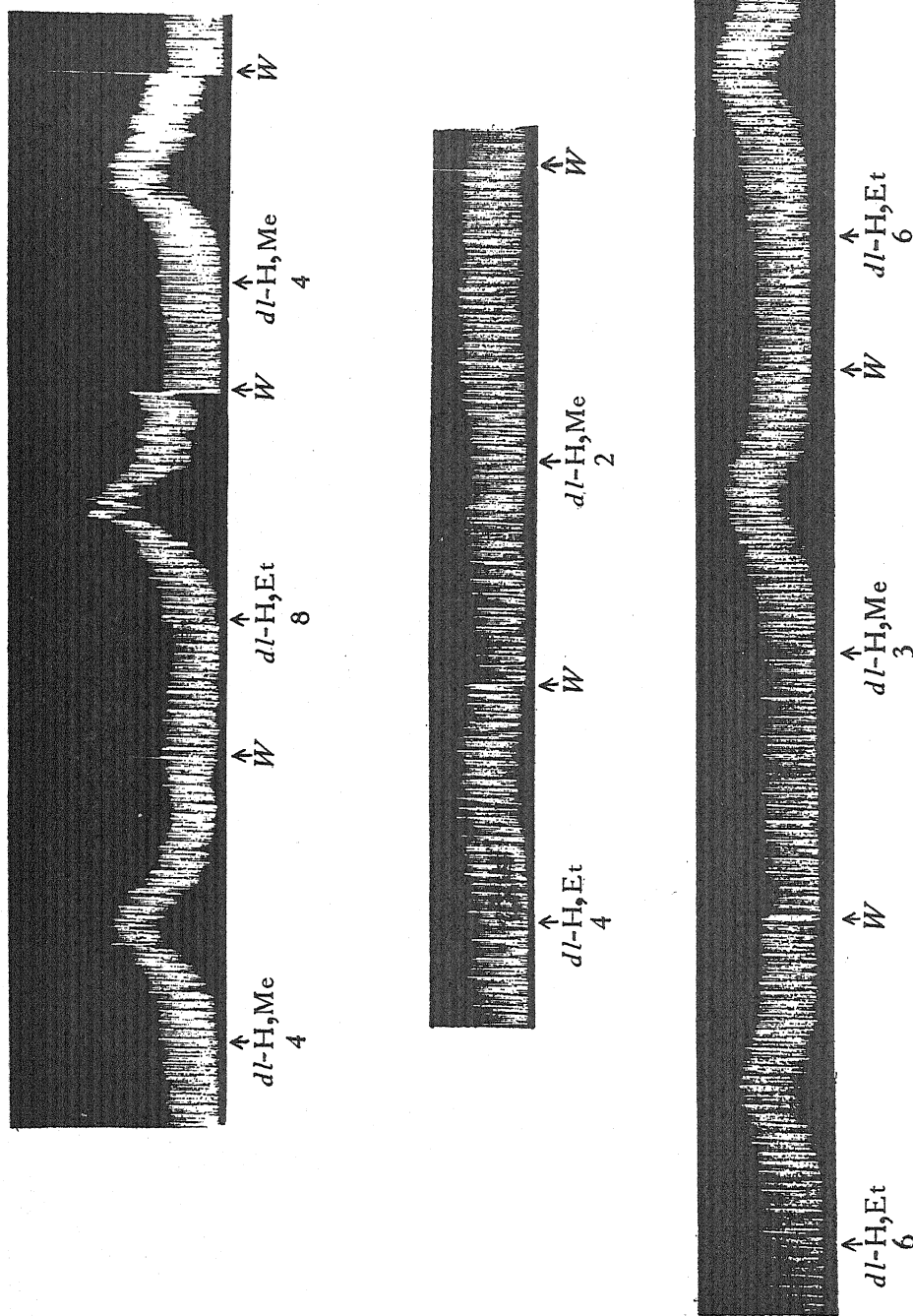


Fig. 2. Continuous tracing showing comparison of the activities of the hydrochlorides of *dl*-niotine (*dl*-H, Me) and of the methylurethane of *dl*- α -*m*-hydroxyphenyl- α -propylmethylaniline (*dl*-H, Et) on the isolated small intestine of the rabbit. Numbers represent parts of drug per 100,000,000 parts of Tyrode's solution. *W* = washed.

the *l*-isomeride has proved to be the more active. In order, however, to obtain numerical data bearing on the subject of the present investigation we have compared the activities of a number of the urethanes mentioned above, including *d*- and *l*-miotine, on the isolated rabbit intestine. The following ratios, which refer to the concentrations required to produce equal action, were obtained for the pairs of urethanes indicated: *dl*-H, Me: *dl*-H, Et = 2:1; Me, Me: *dl*-H, Et = 2.7:1; *l*-H, Me: *d*-H, Me = 5:1; Me, Me: *l*-H, Me = 2:3; *dl*-H, Me: H, H = 12:1. From these figures the relative activities of the various compounds are deduced to be as follows:

H, H	<i>d</i> -H, Me	<i>dl</i> -H, Et	<i>dl</i> -H, Me	Me, Me	<i>l</i> -H, Me
1	5	6	12	16	24

According to the activities thus assigned to *d*- and *l*-miotine, the racemic compound should have an activity of $(24 + 5)/2 = 14.5$. Actually, it was found by direct comparison with the methylurethane of *m*-hydroxybenzyl-dimethylamine to be 12, which is in satisfactory agreement with that calculated.

An inspection of the above figures at once shows that the results obtained are in general accord with that anticipated from our hypothesis. The symmetrical compound Me, Me has an activity which is 3 times that of *d*-miotine and only slightly less (two-thirds) than that of *l*-miotine. Moreover, the diminution in activity due to what we have termed the hydrocarbon effect on passing from miotine to its next higher homologue is, when judged from the compounds *dl*-H, Me and *dl*-H, Et, 6 units. Assuming that the additional methyl group in Me, Me has produced the same effect, the activity for this compound, when corrected by the addition of 6 units, becomes 22, a value which is in satisfactory agreement with that obtained (24) for *l*-miotine. The only discrepancy between these results and our anticipations is the fact that *d*-miotine is 5 times as active as its lower homologue, whereas according to our deductions these substances should be equally active. We do not, however, regard this discrepancy as serious. It is possible that the hydrocarbon effect on passing from H, H to H, Me may be in the opposite direction from that associated with the change from H, Me to Me, Me or H, Et.

In order to illustrate the method adopted, and the degree of accuracy attained, in the comparison of the activities of the different urethanes, the tracings for *dl*-miotine and the methylurethane of α -*m*-hydroxyphenyl-*n*-propyl-dimethylamine (H, Et) are reproduced in Fig. 2. These tracings are typical of those obtained in the experiments recorded above. In each case a strip of the small intestine from the rabbit was suspended in Tyrode's solution and was treated alternately with the two drugs under comparison. It was invariably found that the response of the intestine was at first inconstant but became constant after several hours. At this stage the concentrations of the two drugs under comparison were sought which produced the same effect on the strip of intestine. Only the latter and significant portions of the tracing are reproduced in Fig. 2.

SUMMARY.

1. It has been shown theoretically, on the assumption that three of the groups linked to the asymmetric carbon atom in an optically active drug are concerned in its attachment to its specific receptor in the tissues, that molecular dissymmetry and its associated optical activity have no direct influence on the magnitude of the physiological activity of a drug.

2. The same argument holds with respect to the relationship between molecular dissymmetry in a substrate molecule or in a specific inhibitor and their power of combining with an enzyme.

3. This theory has been shown to be in agreement with the fact that the pressor activities of *d*-adrenaline and 3:4-dihydroxy- β -phenylethylmethanamine are, within the limits of experimental error, equal.

4. A comparison of the miotic activities of a number of urethanes has given results which are also consistent with this theory. Similar results have been obtained in a comparison of the inhibitory activities towards liver esterase and of the activities on the isolated intestine from the rabbit of the same urethanes.

Part of the expense of this investigation has been met by grants from the Earl of Moray Research Fund of this University.

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CLXXI. CYSTINE AND NEPHROTOXICITY.

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(Received July 6th, 1933.)

By administering excessive cystine to rats and other animals many workers appear to have produced injurious effects on the kidneys. This damage has not always been observed however, and it seemed possible that considerable clinical importance might attach to a study of the reason of the variability of the results.

The earlier experiments in which cystine was given to animals were concerned primarily with a study of the fate of ingested cystine. Wohlgemuth [1903-04] appears to have observed no toxic effect in rabbits after oral administration of 5 g. suspended in water. Blum [1904] reports a number of experiments in which cystine was given to dogs in large amounts, and in most of his cases nephrotoxicity was not observed. Abderhalden and Samuely [1905] gave as much as 8 g. of dileucylcystine subcutaneously without producing renal damage. Later, however, Abderhalden [1922] noted that kidney injury accompanied the deposition of crystals of cystine in the renal tubules of the mouse after ingestion of free cystine. Similarly, Lignac [1926] demonstrated that cystine injected in the form of a suspension into mice reappeared in crystalline form in the convoluted tubules. Lewis [1925] introduced 0.5-1.0 g. cystine per kg. in alkaline solution by stomach tube into rabbits every second or third day. After a few doses, there were definite signs of renal injury. Newburgh and Marsh [1925, 1, 2] injected solutions of amino-acids into rabbits and dogs and found that cystine was one of those which were nephropathic. Curtis and Newburgh [1926; 1927] added cystine to the diet of the rat, observing that large doses caused severe nephritis and death in a few days; smaller amounts caused moderate injury in the course of a few months, whilst 1.5 % produced necrosis of the tubules in one year. Cox, Smythe and Fishback [1929] reported acute toxic nephrosis in young rats under 60 g. in weight ingesting 0.3-0.9 % free cystine; some of the animals, however, recovered from their acute illness and proceeded to grow normally without any alteration in the ration. Cox and Hudson [1929-30] extended these observations and obtained evidence that cystine nephrosis might be prevented by increasing the allowance of vitamin B concentrate. The rats exhibited variable resistance to the nephrotoxicity of cystine, and the susceptibility was attributed to hereditary factors rather than to the influence of the stock diet. Addis, MacKay and MacKay [1926], on the other hand, had obtained no evidence of renal impairment from blood-urea determinations, urinary or histological examinations in rats which had lived one-third of their life-time on a diet containing 1 % cystine. The proportion of dried yeast included in their

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diet was 10 %. Recently, Longwell, Hill and Lewis [1932] studied the effect of 0.3-0.6 % of cystine in the diet of young rats receiving either no yeast at all or a 2 g. equivalent of yeast extract. They report "complete absence of evidence that a diet high in cystine produces pathological changes in the kidneys." This was true even of some rats which were in poor condition at the beginning of the experiment. Renal hypertrophy due to absence of yeast was no greater in the presence of cystine.

The insolubility of cystine is a factor which complicated those of the above-mentioned experiments in which cystine was injected parenterally. There are obvious objections to the method of injecting a suspension of cystine or a quantity of an alkaline solution of cystine.

In the matter of the feeding experiments, the positive findings probably cover two states of affairs; there is the acute condition from which, as Cox and co-workers report, the animals recover without change in the ration and the more slowly produced lesions of the type reported by Newburgh.

One question which naturally presents itself is whether the effect of cystine is due to its acidogenic properties. The intention in the experiments herein reported was to determine whether the effects of cystine could be overcome by regulating the proportion of acid to base in the diet. That the quantity of acid produced by the oxidation of cystine is appreciable can be seen by calculating that the addition of 0.1 g. of cystine means a virtual increase of 1.6 cc. of *N* acid. The effect of this increased acidity would be more marked in the case of rabbits than in rats, for the former animals have little power of calling on ammonia to neutralise acids. Arising from the acidogenic qualities of cystine was the question of available base; it was necessary to find out whether the salt mixtures ordinarily employed in compounding synthetic diets were adequate in base content to cover this increased production of acid. The results reported in this contribution were obtained in conjunction with a study of the acid-base mechanism in relation to excessive protein feeding, reported elsewhere [Bell, 1933].

EXPERIMENTAL.

It was decided to attempt to produce the condition of cystine nephrosis in very young rats, such as had been reported by Cox and co-workers, and see whether the addition of potential alkali to the diet prevented its incidence. The diet was compounded of:

					%
"Light white casein"	20
Rice starch	68
Dried brewer's yeast	5
Salt mixture (McCollum's)	5
Cod-liver oil	2

As the quantity of cystine, which in the experience of Cox, Smythe and Fishback was sufficient to induce nephrosis, ranged from 0.3 to 0.9 % of the diet, we began by using 1.0 %, but as no derangement occurred, it was decided very early to raise it to 2.0 %. Even when the rats were only 35 g. in weight when cystine feeding commenced, there was no evidence of cystine nephrosis. The rats were kept in metabolism cages, and the urine was examined daily. One group received enough of a mixture of potassium carbonate (7 parts) and sodium citrate (10 parts) to reduce the acidity of the urine almost to neutrality. The addition of potential alkali did not augment the growth rate—it was already

maximum (Fig. 1). Since no effect from the cystine feeding was obtained, the experiment was terminated at the end of 4 weeks. The fasting blood-urea was determined and was found to be normal in both groups; the average for the animals receiving cystine was 38 mg. per 100 cc.; that for the animals being given alkali as well as cystine was 43 mg. Sections of the kidneys failed to give any evidence whatever of inflammatory changes.

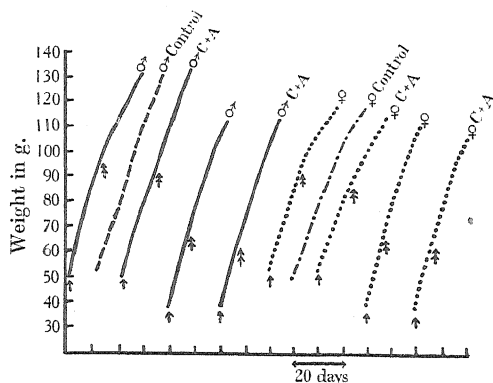


Fig. 1. Average growth curves of rats ingesting 1 and 2 % cystine.

↑ = 1 % cystine in diet;
 ↑↑ = 2 % cystine in diet;
 C + A = Cystine + alkalisating salt.

It is difficult to understand why cystine *per se* should be harmful to the kidneys, unless it is due to insolubility or to acid production. Still more difficult is it to understand why, in spite of its insoluble and acidogenic nature, the animals should recover without change in the ration. If cystine itself exerts a nephropathic action, we should expect to find a high incidence of nephritis in cystinuria, but in the following references in the literature to uncomplicated cases of cystinuria, no mention is made of findings that indicate that the excretory tissue is involved; cystinurics have usually been discovered either accidentally or because of symptoms of renal calculus [Garrod, 1923; Smillie, 1915; Looney, Berglund and Graves, 1923; Lewis and Lough, 1929; Robson, 1929; Brand, Harris and Biloon, 1930]. Certainly, the last-named authors have found that there is excreted, in this disorder of metabolism, a cystine complex which decomposes in the urine with liberation of free cystine; but even so, we should expect that if this liberation of the amino-acid begins early enough to form a stone in the pelvis of the kidney, the process has already begun soon after the formation of the glomerular filtrate.

The variety of kidney affection induced in young rats by excessive cystine feeding seems to be some indirect effect, irregular in its occurrence, and temporary in its influence. The recovery without alteration in the diet seems to suggest that the nephritis is part of an infection. It is possible that the presence of the cystine in the intestine brings about an alteration in the oxidation-reduction potential of the contents of the gut and so favours the growth of anaerobes. Quastel and Stephenson [1926] have demonstrated that the addition of cystine to culture media favours the growth of anaerobes by altering the oxidation-reduction potential. The condition of "pulpy kidney" in lambs seems to have some features in common with cystine nephrosis, and "pulpy kidney" has

recently been ascribed by Bennett [1932] to infection by the *B. ovitoxicus*, an organism allied to the Welch bacillus. If cystine nephrosis is part of an infection in which the obvious and fatal effects are due to kidney involvement, the recovery can be explained by the development of an immunity.

SUMMARY.

Feeding young rats with diets containing 1.0-2.0 % free cystine has failed to produce the condition of "cystine nephrosis." It is suggested that the condition reported by other investigators to occur in young animals is probably due to an infection, to which an impetus is given by the altered oxidation-reduction potential in the intestines.

Grateful appreciation is herewith expressed to the Royal Society of Medicine for the scholarship which has enabled me to make these observations, to the Medical Research Council for defraying the cost of materials and to Prof. J. C. Drummond for helpful advice and criticism.

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CLXXII. ASCORBIC ACID AS THE ANTI-SCORBUTIC FACTOR.

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(Received July 3rd, 1933.)

It is proposed in this short communication to describe some experiments dealing with the antiscorbutic activity of a number of specimens of ascorbic acid (hexuronic acid [Szent-Györgyi, 1928]), its oxidation products and ascorbic acid regenerated from one of the oxidation derivatives. We are indebted to Messrs Burroughs, Wellcome and Co. for the gift of preparations 1, 2, 3, 5 and 7 made in the experimental laboratories of the Wellcome Chemical Works and to Prof. Szent-Györgyi for the gift of preparations 4 and 6.

Preparation 1. This specimen of ascorbic acid was prepared from oranges: C, 41.4, 41.4; H, 5.0, 5.1 %. $C_6H_8O_6$ requires C, 40.9; H, 4.5 %. The m.p. varied with the rate of heating; when placed in a bath at 180° and slowly heated it melted at 182°. 1 mg. decolorised 1.06 cc. N/100 iodine (calc. 1.14). It was tested for antiscorbutic potency in doses of 0.25 mg., 0.5 mg. and 1 mg. prophylactically. The test lasted 50 days, after which time the animals were chloroformed. The doses were weighed out daily and dissolved in air-free water immediately before administration. Table I gives the initial and final weights

Table I.

Dose mg.	Weight (g.)		Remarks
	Initial	Final	
0.25	270	315	—
	282	275	—
	305	335	—
0.5	285	435	—
	270	300	Died after 33 days of intercurrent infection
	305	420	Died after 43 days of intercurrent infection
1.0	270	415	—
	300	465	—
	280	410	Chloroformed after 35 days

of the guinea-pigs on the respective doses. All the animals on the lowest dose showed very marked scorbutic signs at the autopsy. The guinea-pig which survived for 50 days on the 0.5 mg. dose showed slight signs of scurvy at the autopsy, whilst the other two animals on this dose which died before the termination of the test did not show any signs. Two of the animals on the highest dose were free from macroscopic signs of scurvy at the autopsy, the third

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guinea-pig which was chloroformed on the 35th day showed marked haemorrhages in both knee joints but no other signs of scurvy could be discerned.

Preparation 2. This was a crude specimen of ascorbic acid prepared from the adrenal glands of the ox: C, 41.2, 41.1; H, 5.2, 5.1 %: M.P. 184°. 1 mg. required 1.04 cc. N/100 iodine. It was tested in doses of 0.25 mg., 0.5 mg. and 1 mg. prophylactically. The duration of the test was 61 days. A week's requirement of the acid was dissolved in a suitable quantity of air-free water, divided into daily doses and kept at - 20°. The doses were melted just before administration. Table II gives the initial and final weights of the guinea-pigs.

Table II.

Dose mg.	Weight (g.)		Remarks
	Initial	Final	
0.25	265	320	—
	280	290	Died after 36 days of intercurrent infection
	307	267	Died after 54 days of intercurrent infection
0.5	265	440	—
	270	425	—
	270	370	—
1.0	255	420	—
	285	310	Suffered from intercurrent infection which was responsible for fall in weight during the last period of the test
	290	530	—

All the animals on the 0.25 mg. dose showed definite signs of scurvy at the autopsy. Only one guinea-pig on the 0.5 mg. dose showed very slight signs of scurvy; the other two were apparently normal. No macroscopic signs of scurvy could be detected at the *post mortem* examination of the animals on the highest dose.

Preparation 3. This specimen of ascorbic acid was preparation 2 recrystallised four times from methyl alcohol: C, 41.1; H, 4.8 %: M.P. 184° (after first recrystallisation). 1 mg. required 1.06 cc., 1.07 cc. N/100 iodine. It was tested in doses of 0.25 mg., 0.5 mg. and 1 mg. prophylactically. The duration of the test and the technique of administration were the same as for preparation 2. The initial and final weights of the guinea-pigs are given in Table III. At the

Table III.

Dose mg.	Weight (g.)		Remarks
	Initial	Final	
0.25	265	320	—
	280	310	—
	305	295	—
0.5	295	465	—
	270	325	—
	265	430	—
1.0	250	475	—
	290	340	Fall in weight due to pneumonia during last week

autopsy all the animals on the 0.25 mg. dose showed signs of severe scurvy. Of those on the intermediate dose one showed no signs, whilst the other two guinea-pigs showed definite signs of scurvy. The animals on the 1 mg. dose were free from macroscopic signs of scurvy.

Preparation 4. The specimen of ascorbic acid was prepared from adrenal glands and was crystallised from methyl alcohol, ether and light petroleum.

It was analytically pure; M.P. 187° $[\alpha]_{578}^{20} + 24^{\circ}$ in water. 1 mg. required 1.10 cc. N/100 iodine. It was tested in doses of 0.25 mg. and 0.5 mg. prophylactically (duration of experiment 60 days). The technique of the administration of the doses was the same as for preparation 2. The initial and final weights of the guinea-pigs are given in Table IV. All the animals on the lower dose, although

Table IV.

Dose mg.	Weight (g.)		Remarks
	Initial	Final	
0.25	270	295	Pleurisy
	280	240	Died after 48 days from intercurrent infection
	300	365	—
0.5	270	385	—
	280	440	—
	260	360	—

two out of three suffered from intercurrent infection and one actually died of it, showed mild signs of scurvy at the autopsy. Only one guinea-pig out of the three on the 0.5 mg. dose showed a sign of mild scurvy, namely slight discoloration of knee joints, at the *post mortem* examination.

Preparation 5. This was a crude specimen of ascorbic acid from oranges. It was washed as little as possible when separated from the syrup: C, 41.4; H, 5.3 %; M.P. $150-170^{\circ}$. 1 mg. required 0.932 cc. N/100 iodine. It was tested in doses of 0.25 mg., 0.5 mg. and 1 mg. prophylactically. The duration of the test and the technique of administration were the same as for preparation 2. The initial and final weights of the guinea-pigs are given in Table V. The scorbutic signs found at the autopsy of the three animals on the lowest dose were

Table V.

Dose mg.	Weight (g.)		Remarks
	Initial	Final	
0.25	260	250	Died after 49 days
	285	275	Died after 55 days
	280	227	Died after 53 days
0.5	260	350	—
	280	310	—
	290	395	—
1.0	270	450	—
	275	360	—

severe enough to justify the assumption that they died of scurvy. All the guinea-pigs on the intermediate dose also showed marked scurvy after being killed by chloroform. Only one of the guinea-pigs on the 1 mg. dose showed scorbutic signs at the *post mortem* examination. There was no doubt that the condition of the experimental animals on the various doses of this preparation was decidedly worse than that of those on the corresponding doses of the previous preparations. The difference in activity appeared to be greater than would be expected by the fact that this specimen was only about 82 % pure as judged from its iodine titration. Spectrographic examination confirmed this figure.

Preparation 6. This specimen of ascorbic acid prepared from paprika was crystallised from dioxan: M.P. 192° ; $[\alpha]_{578}^{20} + 24^{\circ}$ in water. It was analytically pure. 1 mg. required 1.14 cc. N/100 iodine. In view of the purity of this

specimen it was desirable to obtain the utmost accuracy of which the biological method is capable in assaying its antiscorbutic activity. Ten guinea-pigs were therefore employed on each of the doses of 0.25 mg. and 0.5 mg. The duration of the test was 57 days and in this case the doses were weighed out daily and dissolved in the requisite quantity of air-free water immediately before administration. Table VI gives the initial and final weights of the guinea-pigs.

Table VI.

Dose mg.	Weight (g.)		Remarks
	Initial	Final	
0.25	270	330	Chloroformed after 46 days
	260	280	
	275	270	
	275	380	
	275	275	
	280	330	Died after 52 days of intercurrent infection
	280	235	
	280	270	
	280	285	
	290	390	
0.5	260	385	
	270	330	
	275	435	
	275	410	
	275	410	
	280	435	
	280	405	
	280	390	
	285	370	
	285	480	

All the animals on the lower dose showed definite signs of scurvy at the autopsy; those on the higher dose were free from macroscopic signs of scurvy.

Preparation 7. This was a crude specimen of ascorbic acid prepared from oranges. It had not been recrystallised but separated from the syrup which is obtained at the end of the process and washed with ether: M.P. 178–183°: 1 mg. required 0.980 cc. *N*/100 iodine. Spectrographic examination revealed that it was about 85 % pure. As in the preceding experiment 10 guinea-pigs were employed on each of the doses of 0.29 mg. and 0.58 mg. which, calculated from the iodine absorption of the specimen, should have been equivalent to about 0.25 mg. and 0.50 mg. respectively of the pure acid. The duration of the test and the mode of administration of the doses were the same as in the case of preparation 6. Table VII gives the initial and final weights of the guinea-pigs. All the animals on the 0.29 mg. showed more severe scurvy at the autopsy than the animals on the corresponding dose of the preceding preparation. The condition of the guinea-pigs on the higher dose was also definitely worse than that of the animals on the 0.5 mg. dose of preparation 6 during life and at the autopsy. In seven out of ten of these animals marked signs of scurvy were found at the *post mortem* examination.

Preparation 8. This was the first oxidation product of ascorbic acid. This substance ($[\alpha]_{D}^{20} + 56^\circ$ in dilute HCl) when freshly prepared displays no selective absorption [Hirst, 1933]. It is quantitatively reduced to ascorbic acid by H_2S and HI. The requisite quantity of ascorbic acid (preparation 6), was dissolved in air-free distilled water and *N*/100 iodine added until one drop turned the solution brown. It was then quickly administered to the experimental animals. The growth and other details of the guinea-pigs on this preparation, as those of the animals on preparation 2, are represented in Fig. 1. It is seen that the

preparation is only slightly less active than ascorbic acid which had not been previously oxidised. The graphs of growth of the animals on preparation 2

Table VII.

Dose mg.	Weight (g.)		Remarks
	Initial	Final	
0.29	255	365	—
	255	215	Died after 54 days of scurvy
	260	290	—
	260	310	—
	260	250	Died after 46 days. Severe scurvy. No complications
	265	380	—
	270	365	—
	270	310	—
	275	310	Died after 42 days. Severe scurvy. Intestinal infection
	285	305	Died after 54 days of scurvy complicated by pneumonia
0.58	255	480	—
	255	430	—
	260	345	Died after 46 days of pneumonia. No scurvy
	260	395	—
	260	410	—
	265	430	—
	270	485	—
	270	275	Died after 58 days of scurvy complicated by pneumonia
	270	390	—
	280	425	—

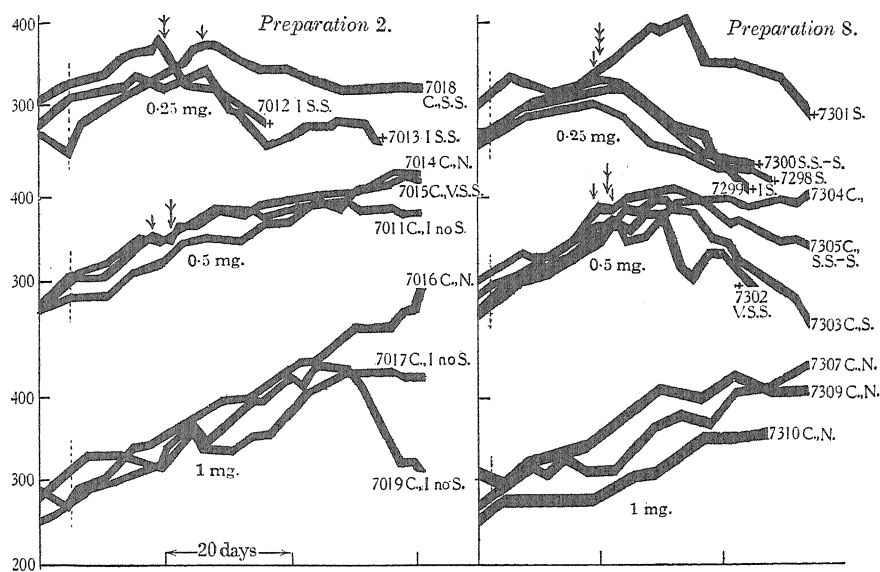


Fig. 1.

C. = chloroformed
+ = died
S. = scurvy
S.S. = slight scurvy
V.S.S. = very slight scurvy
N. = normal
no S. = no scurvy
I = intercurrent infection
= = beginning of dosing
↓ = onset of clinical symptoms of scurvy

were used for comparison because the animals on the 0.25 mg. and 0.5 mg. doses of this specimen behaved in the same way as the corresponding animals on preparation 6; in addition a 1 mg. dose was also used in this test. Demole [1933]

found that an oxidation product from ascorbic acid (*Dehydroascorbinsäure*) was somewhat less active than ascorbic acid. He does not however give any experimental details, and it is difficult to judge whether he was dealing with the above oxidation product.

Preparation 9. When chlorine-water is added to a concentrated solution of ascorbic acid until the calculated amount of chlorine (2 atomic proportions) has been taken up the specific rotation becomes $[\alpha]_{578}^{20} + 56^\circ$ (calculated as ascorbic acid). If this solution is kept at room temperature the rotation gradually diminishes to the value $[\alpha]_{578}^{20} - 6^\circ$, equilibrium being reached in about 60 hours. Spectrographic examination shows the complete absence of the ascorbic acid band from the spectrum of the equilibrium oxidation product. It does not react with iodine in acid solution nor does it decolorise indophenol. It reduces ammoniacal silver nitrate and alkaline sodium hypiodite [Cox *et al.*, 1932; Herbert and Hirst, 1932; Hirst, 1933; Hirst *et al.*, 1933]. On reduction with HI it gives an approximately 80 % yield of ascorbic acid. With H_2S however the yield is only about 10 %. The solutions of the equilibrium product used for the biological tests contained about 0.3 g. of the oxidation product per 100 cc. and sufficient HCl (0.5 mol. per mol. ascorbic acid) to render the compound stable. Repeated chemical tests showed that such solutions were perfectly stable over periods of several months. It was tested in doses equivalent to 0.25 mg., 0.5 mg. and 1 mg. with four animals on each dose. The guinea-pigs on the first two doses behaved as if they were on a basal diet alone and died of typical scurvy in about a month. Three animals on the highest dose died from scurvy after 42 days and one after 29 days. There was in the specimen, therefore, but little antiscorbutic activity as compared with the ascorbic acid from which it was prepared.

Preparation 10. This consisted of ascorbic acid obtained by reduction of the first oxidation product. Ascorbic acid in dilute aqueous solution was oxidised by iodine (2 atomic proportions, dissolved in the minimum quantity of alcohol). The solution now contained the first oxidation product, $[\alpha]_{578}^{20} + 56^\circ$, together with 2 mols. of HI liberated during the reaction. The solution was slowly evaporated to dryness at room temperature in a vacuum desiccator. Regeneration of crystalline ascorbic acid then took place, with separation of iodine, which was removed by volatilisation at room temperature. A small amount of brown amorphous material was formed as a by-product. The yield (75–90 %) of ascorbic acid depended on the time which elapsed between oxidation and regeneration, freshly oxidised material giving the highest and the "equilibrium" material the lowest. The regenerated ascorbic acid was identical chemically with an authentic sample.

Three specimens prepared at different times and under different conditions were utilised. One, which was administered during the first 24 days, was recrystallised ascorbic acid regenerated from a sample of oxidised material kept for 3 days before reduction. The second sample, used from the 25th to the 40th day, had not been recrystallised and the solution from which it was obtained had been kept for 12 hours before being evaporated. The third sample used from the 40th to the 52nd day was prepared in the same way as the second.

It will be seen from Fig. 2 that the response of the guinea-pigs to this preparation was similar to that obtained with other specimens of ascorbic acid. It must however be pointed out that the animals on the 1 mg. dose, unlike those animals on the corresponding dose of the other preparations of ascorbic acid, showed unmistakable symptoms of scurvy after 24–29 days. These symptoms persisted until death although macroscopic signs of the disease were not found at the *post mortem* examination.

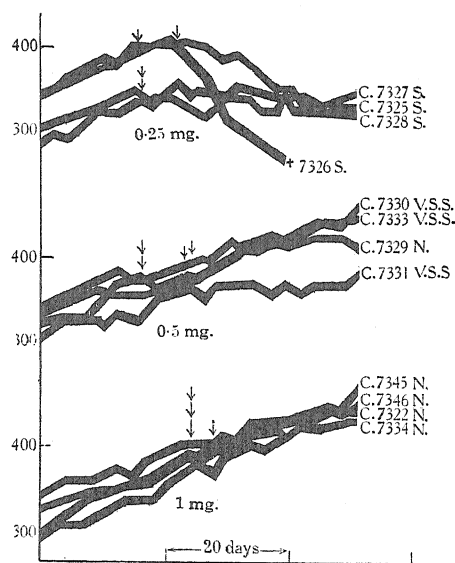


Fig. 2.

Symbols have same significance as in Fig. 1.

CONCLUSIONS.

A great disparity in the antiscorbutic activity of specimens of ascorbic acid originating from different natural sources and possessing different degrees of purity might throw serious doubts on the identity of ascorbic acid with vitamin C. On the other hand, in view of the limitations of the biological method and the persistent association of some physiological principles with chemically known substances the constant antiscorbutic activity of ascorbic would not constitute in itself sufficient proof of its identity with vitamin C. The activity of preparation 10, however, offers much more decisive evidence in this respect. This substance, which showed a potency of the same order as the other samples of ascorbic acid, was obtained by regeneration from an oxidised preparation. A similar oxidised preparation (preparation 9) was found scarcely active in doses as high as 1 mg. per diem. We have so far been precluded from comparing the antiscorbutic potency of an equilibrated oxidation product and the ascorbic acid regenerated from the same specimen. This we hope to accomplish in connection with the chemical and biological study of this interesting product which is now in progress. We are, however, strongly of the opinion that all equilibrated oxidation products are, weight for weight, much less active than the ascorbic acid regenerated from them. Three specimens regenerated at different times were used in this prophylactic test at intervals. A marked deviation in activity of any of these specimens would have manifested itself in the condition of the animals, especially of those on the lower doses. It therefore appears that ascorbic acid is active *per se*. The alternative explanation that vitamin C is associated with ascorbic acid and like it is reversibly oxidised and regenerated quantitatively is much less plausible.

Of further interest is the observation that ascorbic acid, when tested immediately after oxidation with iodine, shows little loss in antiscorbutic activity. This observation is similar to the one made by Zilva [1927] on decitrated lemon

juice oxidised with phenolindophenol. The explanation offered at the time by him was that an accompanying reducing substance which exercised a protective function towards the vitamin in the juice was directly oxidised and not the vitamin itself. Tillmans, Hirsch and Siebert [1932], confirming this observation, offered another explanation, namely, that the vitamin whilst still retaining most of its activity was reversibly oxidised. They were able to show that decitrated lemon juice oxidised with 2:6-dichlorophenolindophenol or iodine could regain its reducing capacity on reduction with hydrogen sulphide [Tillmans, Hirsch and Dick, 1932]. This has recently been confirmed by Johnson [1933]. The above observation on the retention of the antiscorbutic potency of the reversible oxidation product of ascorbic acid suggests therefore that Tillmans's hypothesis is the correct one. It is interesting to note in this connection that there is a parallelism between the antiscorbutic activity of the oxidised ascorbic acid and its capacity of being regenerated by hydrogen sulphide.

Attention has been drawn to the fact that some specimens of ascorbic acid (preparations 5, 7 and 10) showed somewhat lower activity than the others. This difference in potency, if real and not due to traces of interfering substances—all these specimens were crude—cannot at present be explained.

We are indebted to Dr R. W. Herbert for assisting us in some of the experiments.

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CLXXIII. THE SILVER NITRATE STAINING REACTION FOR ASCORBIC ACID IN THE ADRENAL, PITUITARY AND OVARY OF VARIOUS SPECIES OF ANIMALS.

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(Received May 31st, 1933.)

SZENT-GYÖRGYI [1928] has demonstrated that when a section of the adrenal of the ox is immersed in 0.4 % AgNO_3 solution, its cortex darkens owing to the deposition of metallic silver. The reduction, he concluded, was due to the presence of ascorbic acid (hexuronic acid). In view of the recent observations on the high and persistent antiscorbutic activity of ascorbic acid it became obvious that the above observation could be utilised in studying the function of ascorbic acid in the development of scurvy. The experiments to be described here were originally undertaken by Zilva with the hope of obtaining information concerning the problem of the identity of ascorbic acid with vitamin C. In this respect suggestive but not conclusive evidence has so far been obtained from this investigation, but some facts have emerged which we consider of sufficient interest to be put on record.

In preliminary experiments guinea-pigs on a scorbutic diet receiving various doses of decitrated lemon juice and others subsisting on a diet containing cabbage *ad lib.* were examined. It transpired that although the cortex of the adrenals from the animals receiving cabbage *ad lib.* darkened markedly on immersion in the AgNO_3 , this was not so in the case of the adrenal glands from animals which were receiving daily doses of as much as 10 cc. of decitrated lemon juice, although the guinea-pigs were found at the *post mortem* examination to be in excellent condition and free from scurvy. Even the microscopical examination of sections of the glands treated with AgNO_3 did not reveal any deposition of Ag in the adrenal cortex of the guinea-pigs protected with high doses of decitrated lemon juice. Yet histological sections of the adrenals from these animals did not show any abnormality.

The search when extended to other organs from various species of animals, irrespective of their susceptibility to scurvy, revealed that certain other organs showed the AgNO_3 staining reaction macroscopically, especially the anterior and intermediate lobes of the pituitary gland, which in some species reduced the reagent even more markedly than the cortex of the adrenal. The disparity in the intensity of staining in these two glands was particularly marked in the human subject, where with very few exceptions the anterior lobe of the pituitary

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was found to stain more intensely than the adrenal cortex. In the case of the ox the anterior lobe of the pituitary was moreover found to be antiscorbutically very active. As had been found in the guinea-pig, in many of the human cases examined the adrenal cortex did not stain, although the patients were free at least from clinical symptoms of scurvy.

EXPERIMENTAL.

Technique.

The scorbutic diet employed in these experiments was similar to that already described [Bracewell, Hoyle and Zilva, 1930] and was complete in all essentials apart from vitamin C. Freshly prepared decitrated lemon juice was administered daily, except during the week-end, allowance being made for this shortage during the remaining days of the week. The guinea-pigs on the mixed diet received oats, bran and cabbage *ad lib*.

The silver staining was carried out in the dark since the gland reticulum stains in the light independently of the presence of ascorbic acid. The slices were placed in 0.4 % AgNO_3 solution for 15 minutes, after which time they were fixed in a 5 % solution of sodium thiosulphate for 10 minutes. They were then washed in several changes of distilled water and stored in 50 % alcohol. The assessment of the intensity of the staining was carried out with the help of a hand lens. For the microscopical examination the material after treatment with absolute alcohol for 3 hours was cleared and embedded in paraffin, and serial sections were made without further staining.

For general histology, sections fixed in formalin and embedded in paraffin were stained with haemalum and eosin. Fat was examined in frozen sections stained with Sudan III and haemalum. The chrome reaction was carried out by Ogata's method [1917], and Gough and Fulton's mitochondrial method [1929] was used in the cytological examination.

The silver nitrate staining reaction of the organs of various species of animals.

Man. Sometimes there was some staining in the cortex of the adrenals but it was in most cases comparatively slight. In several cases there was distinct staining of the medulla even when the cortex showed none at all. The anterior lobe of the pituitary stained more markedly than the adrenal. Staining sometimes occurred in the ovary, and in one or two cases it was confined to the linings of the larger follicles. The corpus luteum of pregnancy in two cases showed uniform but slight staining.

Ox. The cortex of the adrenals stained markedly. In fresh glands there was very little staining of the medulla but in glands from animals killed about 3 to 6 hours previously the medulla also stained appreciably. The anterior lobe, pars intermedia and cone of Wulzen of the pituitary stained very darkly. In cases in which the adrenals and pituitary from the same animal were compared, the latter almost invariably stained the more intensely. In the ovary (two heifers) there was slight staining of the corpora lutea, but not in the rest of the gland. In the testicle (one animal) there was slight staining.

Dog (5 animals). In the adrenal the cortex and the medulla stained deeply. The anterior lobe and the pars intermedia of the pituitary stained very intensely. The corpora lutea of the ovaries also stained quite darkly.

Cat (2 animals). Staining of the adrenal cortex and of the anterior lobe of the pituitary was well marked. In one animal the staining of the two organs

was of about equal intensity, and in the other the pituitary was slightly darker than the adrenal. The adrenal medulla showed only slight staining in each case.

Guinea-pig. The adrenal stained quite darkly in most instances. The anterior lobe of the pituitary also showed definite staining which in some instances was equal to that of the adrenal but in others was somewhat less. The ovaries showed a slight diffuse staining. The staining reaction in this animal will be dealt with more fully in the next section.

Rat. The cortex of the adrenal stained very intensely. In only one out of five animals there was slight staining found in the medulla. Staining of the adrenals was also found in rats which had subsisted for about 8 weeks on a diet free from vitamin C [cf. Moore and Ray, 1932]. The pituitary also showed distinct staining but it was never quite as dark as that of the adrenal. The ovary showed slight diffuse staining.

It is to be pointed out that, contrary to expectations, in no single case did the livers from the above species of animals stain with silver nitrate.

The silver nitrate staining properties of the adrenal glands of guinea-pigs protected from scurvy with decitrated lemon juice.

A few representative cases will be described in this connection. In the earlier experiments (B 24, B 44 and B 23) the adrenal glands were not examined microscopically for silver staining but only by means of a lens. They are included here because some of these guinea-pigs were on their respective diets for a number of months. Moreover the assessment by the macroscopical method was found to be as reliable as the microscopical examination of sections. Although the medulla of the adrenal also stained in the animals receiving cabbage *ad lib.* the staining never approached that of the cortex in intensity.

B 24. This animal subsisted on a scorbutic diet and a daily dose of 1.5 cc. of decitrated lemon juice from December 8th, 1931, to December 31st, 1932, when it was killed by chloroform: initial weight 290 g.; final weight 515 g. At the autopsy, apart from slight ridging at one of the costochondral junctions, no macroscopic signs of scurvy were to be observed. The adrenal glands did not stain with AgNO_3 . The histological examination of sections stained with haemalum and eosin revealed that there was deep congestion but no other abnormality. These glands were however somewhat enlarged.

B 44. This animal subsisted on a scorbutic diet and a daily dose of 10 cc. of decitrated lemon juice from March 3rd, 1932, to September 21st, 1932, when it was killed by chloroform: initial weight 260 g.; final weight 660 g. No macroscopic signs of scurvy were observed at the autopsy. The adrenals did not stain with AgNO_3 . The histological examination revealed nothing abnormal.

B 23. This animal, which subsisted on a mixed diet containing cabbage *ad lib.*, weighed 650 g. when chloroformed, about the same weight as B 44. Its adrenal cortex however stained very darkly with AgNO_3 . The histological appearance of the glands was quite normal.

B 33. This animal subsisted on a scorbutic diet and a daily dose of 10 cc. of decitrated lemon juice from April 15th, 1932, to March 1st, 1933, when it was chloroformed: initial weight 265 g.; final weight 600 g. There were no macroscopic signs of scurvy at the autopsy. The staining of the adrenals with AgNO_3 was so slight that it could not be perceived with the naked eye although a trace could be discerned with a lens. Histological and cytological appearances and the chrome reaction were normal. The pituitary did not stain with AgNO_3 .

B 34. This animal subsisted on a scorbutic diet and a daily dose of 10 cc. of decitrated lemon juice from June 6th, 1932, to March 1st, 1933, when it was

chloroformed: initial weight 375 g.; final weight 560 g. There were no macroscopic signs of scurvy at the autopsy. The adrenals did not stain with AgNO_3 . Histological and cytological appearances and the chrome reaction were normal. AgNO_3 staining of the pituitary could only be observed with the help of a lens.

B 58. This animal subsisted on a scorbutic diet and a daily dose of 10 cc. of decitrated lemon juice from January 3rd, 1933, to March 1st, 1933: initial weight 300 g.; final weight 340 g. There was a check in the growth of the animal during the first month, which was apparently due to an intercurrent disease from which the guinea-pig recovered. There were no macroscopic signs of scurvy at the autopsy. The adrenals did not stain with AgNO_3 . Histological and cytological appearances and the chrome reaction were normal. The AgNO_3 staining of the pituitary could only be observed with the help of a lens.

B 59. The dietetic history of this animal is the same as that of *B 58*: initial weight 285 g.; final weight 470 g. There were no macroscopic signs of scurvy at the autopsy. As in *B 34* the AgNO_3 staining was so slight that a trace could be discerned only with the aid of a lens. Histological and cytological appearances and the chrome reaction were normal. The AgNO_3 staining of the pituitary could only be observed with the help of a lens.

B 17. This animal, which subsisted on a mixed diet with cabbage *ad lib.* and weighed 720 g. when chloroformed, was used as a control for *B 33*, *B 34*, *B 58* and *B 59*. The cortex of the adrenal gland and the anterior lobe of the pituitary from this animal, in contradistinction to the aforementioned animals, stained very darkly. The histological and cytological appearances and the chrome reaction were normal as in the case of the guinea-pigs which received decitrated lemon juice.

The adrenals from guinea-pigs in advanced stages of scurvy did not stain with silver nitrate [*cf.* Moore and Ray, 1932; Miller, Siehrs and Brazda, 1933; Siehrs and Miller, 1933] but, unlike the above specimens, they deviated from the normal macroscopically and especially histologically, showing gross haemorrhages and foci of necrosis.

The absolute diameter of the fat zone of the adrenals showed no appreciable differences in the above animals (Table I). In *B 17*, *B 33* and *B 34* there was

Table I.

No. of specimen	Diam. cortex mm.	Diam. fat zone mm.	Fat content of cortex
			%
<i>B 17</i>	1.9	0.86	45
<i>B 33</i>	1.2	0.83	70
<i>B 34</i>	1.4	0.82	59
<i>B 58</i>	0.96	0.7	73
<i>B 59</i>	0.99	0.7	71

no abnormality in the appearance of the sections stained with Sudan III. In *B 58* and *B 59* the number of large fat droplets was greater than in the control glands of comparable size. The cells, however, were not distended, and the total amount of fat was only slightly greater than in the normal.

The antiscorbutic activity of the anterior lobe of the pituitary of the ox.

The very marked silver nitrate staining capacity of the anterior lobe of the pituitary suggested the necessity of assessing the antiscorbutic potency of this gland. The fresh pituitary from the ox which was always found to stain very darkly was therefore tested. Fig. 1 gives the growth curves and clinical signs

of guinea-pigs which received daily 0.25 g. of the anterior lobe of the gland, and for comparison those of guinea-pigs which received daily doses of 0.25 g. and 0.5 g. respectively of adrenal cortex from the ox [Zilva, 1932]. It will be seen that the condition of the animals on the 0.25 g. dose of the pituitary was as good as that of the guinea-pigs on the 0.5 g. dose of the adrenal cortex. The *post mortem* findings in these two groups were also similar. The pituitary was therefore found to be twice as active as the adrenal cortex. As the glands originated from different animals of unknown dietetic history it is impossible

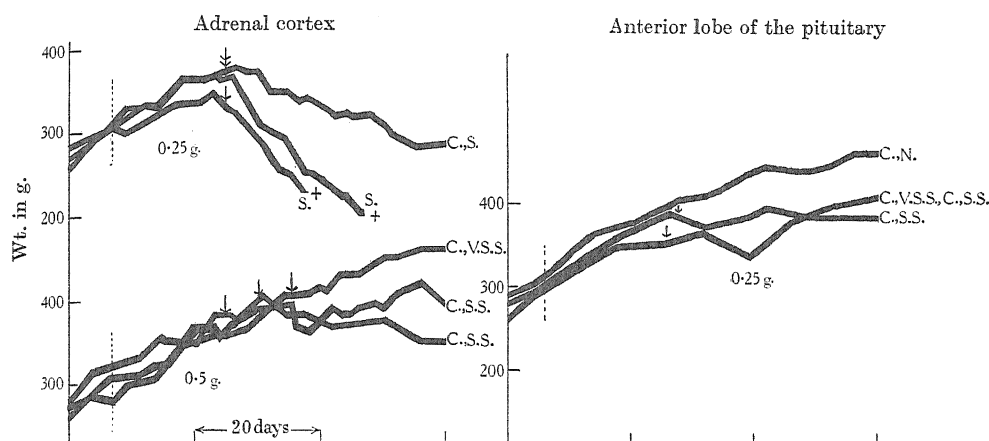


Fig. 1.

⋮ = Beginning of dosing.
C. = Chloroformed.
+ = Died.
S. = Scurvy.

↓ = Onset of clinical symptoms of scurvy.
S.S. = Slight scurvy.
V.S.S. = Very slight scurvy.
N. = Normal.

to say whether this relative antiscorbutic activity holds true always in the case of the ox. It is, however, definitely established that the anterior lobe of the pituitary, which when compared with decitrated lemon juice contains 40 to 50 international units of vitamin C per g. of fresh tissue, can be as potent or rather more potent than either of the two most active natural sources on record, namely, the Alphonso variety of mango [Perry and Zilva, 1932] and paprika [Svirbely and Szent-Györgyi, 1933]. It may be mentioned here that Agnoli [1932] did not find any antiscorbutic activity in desiccated pituitary glands.

The relative silver nitrate staining capacity of the adrenal and pituitary glands in man.

Reference was made in an earlier section to the fact that the anterior lobe of the pituitary in man stains more intensely than the adrenal cortex. This observation is brought out in detail in Table II. The dietetic history of the patients is rather vague since it was difficult to gauge the patients' daily food intake from the details that could be obtained, especially as regards vitamin C during the few months preceding death. Nevertheless it is striking that out of 42 patients, none of whom manifested clinical symptoms of scurvy, 23 showed hardly any silver nitrate staining (— or ±) in the adrenals and the highest score recorded for this gland was only ++. The anterior lobe of the pituitary on the other hand with very few exceptions always stained more intensely. In some

Table II.

No.	Age	Sex	Disease	Diet	Nutrition	Staining		Time of autopsy after death hours
						Pituitary	Adrenal	
37	55 years	F	Pernicious anaemia	Fluids	Fairly good	+	—	—
38	9 "	F	Cerebral abscess	Mixed diet	Good	+++	++	7
39	34 "	M	Chronic meningitis. Hydrocephalus	Mainly fluids	Good	++	±	14
40	71 "	M	Enlarged prostate. Pulmonary embolism	Light mixed	Very good	++	+	14½
41	5 days	F	Bronchitis	—	Good	++	+±	24
42	5 years	F	Strangulation of intestine	Mixed diet	Fair	+++	—	57
43	55 "	M	Papilloma of bladder. Bronchopneumonia	Light mixed diet	Good	+	++	12
44	50 "	M	Carcinoma of pancreas	Mainly fluids	Very poor	±	—	3
45	45 "	F	Obstructive jaundice	Fluids	Very poor	±	—	38
46	57 "	M	Gastric ulcer. Pulmonary embolism	Fluids then light diet	Poor	±	—	19
47	45 "	M	Carcinoma of oesophagus	Fluids	Poor	+	—	18
48	67 "	F	Cerebral softening	Mixed diet	Good	++	—	10
49	1½ "	F	Tuberculous peritonitis and meningitis	Fluids	Very poor	—	—	9
50	12 "	M	Stenosis of oesophagus	Fluids	Very poor	±	++	5½
51	8 "	M	Mastoiditis. Meningitis	Fluids	Fair	++	±	38
52	56 "	M	Carcinoma of lung. Metastasis in brain	Light mixed diet	Good but had lost much weight	+	—	18
53	50 "	M	Ethmoiditis. Septic meningitis	Mixed diet then fluids	Poor	—	—	45
54	62 "	M	Carcinoma of stomach	Light mixed diet. Fluids for 1 week	Fairly good	++	—	16
55	16 hours	F	Laceration of tentorium cerebelli	—	Fairly good	++	±	28
56	43 years	F	Pneumonia. Strangulation of intestine	Fluids	Fair	+	Trace	33
57	0	F	Still born	—	Full term. Weight 8½ lbs.	++	+	24
59	1½ years	F	Tuberculous meningitis	Fluids	Good	+++	++	30
60	32 "	F	Abortion. Infection of uterus	—	Good	+++	++	15
61	42 "	F	Gummatous meningitis	Mixed diet	Obese	++++	±	10
62	14 "	F	Congenital hydrocephalus. Bronchopneumonia	Mixed diet	Fair	+	—	6
63	49 "	F	Myeloid leucaemia	Light mixed diet	Fairly good	++	—	22
64	1 day	F	Prematurity	—	Weight 5 lbs.	++	±	43
65	37 years	F	Acute myeloid leucaemia	—	Good	++++	+	6
66	33 "	M	Tuberculous pericarditis	Mixed diet	Good	+++	±	7½
67	42 "	M	Carcinoma of adrenals. Metastasis in brain	—	Fairly good	++	Involved in tumour	36
68	47 "	M	Cholecystitis. Duodenal ulcer	Light mixed diet	Very good	++	±	11
69	36 "	F	Gangrenous cystitis following pregnancy	—	Fairly good	+++	++	22
70	75 "	M	Carcinoma of colon	—	Very good	—	+	61
72	35 "	M	Malignant melanoma. Metastases in liver	—	Very poor	±	—	6
74	24 "	M	Cellulitis of face. Septic meningitis. Pyaemia	Mixed diet then fluids	Good	+±	+	27

STAINING REACTION OF TISSUES FOR ASCORBIC ACID 1285

Table II (*contd.*).

No.	Age	Sex	Disease	Diet	Nutrition	Staining		Time of autopsy after death hours
						Pituitary	Adrenal	
75	5 months	F	Convulsions. Rickets	Condensed milk	Good	Trace	—	16
76	4 years	M	Pneumonia. Empyema	—	Fair	+	+	40
77	18 "	M	Abscess of lung. Empyema	—	Very poor	+	+	12
78	4 "	M	Sarcoma of jaw. Bronchopneumonia	Fluids	Poor	+	Trace	9
79	48 "	F	Obstructed labour. Uterine infection	Mixed diet	Obese	+++ at periphery + at centre	+	18
80	31 "	M	Abscess of liver. Peritonitis	Mixed diet then fluids	Fair	+++	+	—
81	25 "	F	Puerperal septicaemia	Mixed diet then fluids	Very good	++	+	36

— No staining.
 ± Slight staining barely perceptible to naked eye but recognised with lens.
 + Staining recognisable distinctly to naked eye giving greyish appearance.
 ++ Definite staining.
 +++ Marked staining.
 ++++ Extremely dark staining.

cases the disparity was extremely marked (42, 61, 65, 66, 79 and 80). It can hardly be explained by the fact that the adrenal is more perishable than the pituitary, since it occurs in some cases when the autopsy was carried out 6 and 10 hours after death (Nos. 61 and 65). Moreover, appreciable staining of the adrenal cortex was obtained in certain other cases when the period was longer (Nos. 59, 60, 69). It is also of interest to note that the reducing substance was present in the pituitary of a full term infant which failed to breathe (No. 57) and also in a premature (35 weeks) infant which lived 6 hours (No. 64).

CONCLUSIONS.

On the assumption that the substance reducing silver nitrate is ascorbic acid it would appear from the preceding experiments that it occurs in at least three tissues, namely, adrenal, pituitary and ovary of all animals whether they are susceptible to scurvy or not. It also transpires that the human subject and the guinea-pig may be free from any scorbutic symptoms and yet not show the presence of ascorbic acid in any of these organs by the method employed. In the case of the guinea-pig it has been demonstrated that even when the animals received a dose of decitrated lemon juice very much above the minimum protective dose for three months or longer no accumulation of ascorbic acid could be discovered by this method in the adrenal cortex or pituitary. If the absence of staining be an index of the total absence of ascorbic acid the experiments would suggest that its presence in these glands cannot be essential. The concentration of the reducing substance and of vitamin C in the adrenals and in the anterior lobe of the pituitary is rather striking since these two glands are physiologically related. Our present knowledge of this relationship is, however, too vague to justify us in attaching much significance at this stage of the inquiry to this apparently outstanding selective capacity for ascorbic acid. It is further shown that there is a rough parallelism between the intensity of staining and antiscorbutic activity in the anterior lobe of the pituitary gland

as well as in the adrenal cortex. On the other hand the liver, which contains vitamin C, although in smaller quantities than the above glands, does not stain with silver nitrate.

SUMMARY.

1. The adrenal cortex, anterior and intermediate lobes of the pituitary and the ovary from various animals, whether susceptible to scurvy or not, stain with silver nitrate in the dark. The liver, on the other hand, does not stain with this reagent.

2. The adrenals from guinea-pigs subsisting on a scorbutic diet *plus* high doses of decitrated lemon juice do not stain with silver nitrate although the animals are fully protected from scurvy and show no abnormalities macroscopically and microscopically at the autopsy.

3. The anterior lobe of the pituitary from the ox was found to be extremely potent antiscorbutically (40-50 international units of vitamin C per g. of fresh tissue).

4. The examination of the adrenals and pituitary glands from 42 human cases free from clinical scurvy revealed that, although in the majority of these cases the adrenals did not stain with silver nitrate, the anterior lobe of the pituitary did so in most cases; the intensity of staining being almost always more marked in the latter gland.

We should like to take this opportunity of expressing our indebtedness to Messrs Burroughs, Wellcome and Co. for the gift of the pituitary gland and to Miss O. E. V. Perry for helping with the tests.

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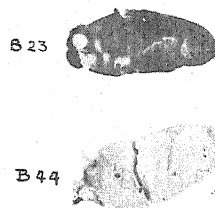


Fig. 1. B 23, B 44. Stained 15 mins. in 0.4 % AgNO_3 .

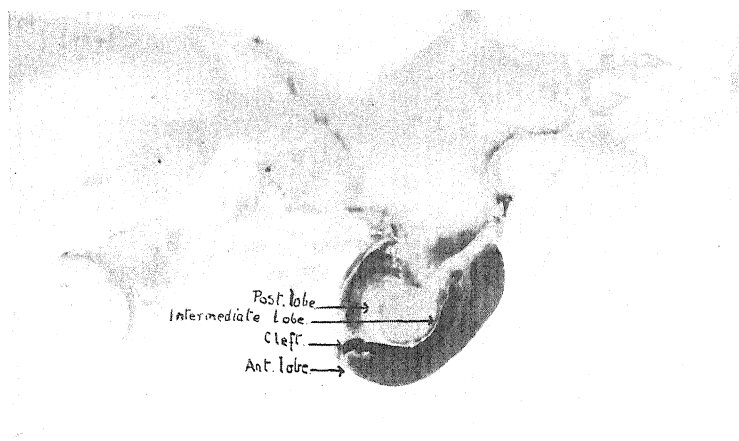


Fig. 2. Pituitary of dog. Stained 15 mins. in 0.4 % AgNO_3 . Showing intense staining of anterior lobe.

CLXXIV. THE REGENERATION OF THE REDUCING PROPERTIES OF OXIDISED LEMON JUICE.

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(Received July 3rd, 1933.)

TILLMANS *et al.* [1932] concluded, as a result of their work upon the relation between the indophenol-reducing capacity and antiscorbutic potency of fruit and vegetable juices, that these properties could be attributed to the same substance, which was most probably identical with Szent-Györgyi's hexuronic acid. These workers claimed that the capacity of lemon juice for reducing indophenol, after being destroyed by oxidation with this reagent, iodine or hydrogen peroxide, could be quantitatively regenerated by reduction with hydrogen sulphide, providing that the reduction was carried out soon after the oxidation and the oxidised juice was not exposed to air too long; that is to say, the oxidation of the reducing substance proceeded first to a stage from which it could be regenerated. Szent-Györgyi had previously claimed [1928] that hexuronic acid (ascorbic acid) was capable of being reversibly oxidised and reduced. It is the purpose of this note to record a few experiments which confirm these observations on the regeneration of the reducing capacity of lemon juice after oxidation with certain reagents.

In repeating the work of Tillmans *et al.*, various difficulties were encountered before their results could be substantiated. In the first place it was found necessary to use juices in which no traces of iron were present as this vitiated the results. The major difficulty, however, was in completely removing the hydrogen sulphide, which, of course, reduces both indophenol and iodine. The above workers claimed that they were able to remove the hydrogen sulphide in about 40 minutes by bubbling nitrogen through the juice. They tested their juices for its absence by dipping lead acetate papers into the solution. It was found in the experiments described below, however, that after passing nitrogen for about an hour, although the presence of hydrogen sulphide could not be detected by dipping lead acetate papers or by allowing the escaping gas to impinge upon wet lead acetate paper, the use of dry reaction paper revealed the presence of a considerable quantity of hydrogen sulphide in the solution. Furthermore, after bubbling nitrogen for 2 hours, the issuing gas also decolorised a solution of indophenol when bubbled through it. Using a dry paper, hydrogen sulphide could be detected even after passing nitrogen for 5-6 hours. Decitrated lemon juice gives a heavy precipitate with lead acetate; it is thus probable that the test for the presence of hydrogen sulphide would be vitiated by this precipitation when the paper was dipped in the liquid. Complete removal of hydrogen sulphide however was not necessary, for it was found that after passing nitrogen for about 3 hours, except in the case of oxidations with hydrogen peroxide, the persistent residuum of hydrogen sulphide was insufficient to affect

appreciably the titrations. The juices were considered to be free from hydrogen sulphide when the issuing gas failed to decolorise a solution containing 1 cc. $N/1000$ indophenol when bubbled through it for half an hour.

The ratio between the capacity of lemon juice for reducing iodine in acid solution and indophenol in neutral solution was almost invariably constant, and the amounts of these reagents reduced were roughly equivalent. Good agreement between indophenol and iodine titrations of the oxidised and untreated juices, and the absence of hydrogen sulphide by the above test, were therefore regarded as criteria that regeneration had been effected.

Experiments in which indophenols were used as oxidising agents gave rather low results after regeneration, but this was probably due to the relatively high concentration of the reduced form of the indophenol which very rapidly absorbs oxygen and may have catalysed the oxidation of part of the reducing substance before it could be titrated [see Zilva, 1927]. In these experiments also no iodine titrations could be undertaken as the indicator interfered.

The experiments with iodine as oxidising agent gave the most satisfactory results both with decitrated lemon juice and the raw juice.

Since hydrogen peroxide functions most satisfactorily in an acid medium, oxidations with this reagent were performed on the raw juice.

Some typical experiments and results are given below.

EXPERIMENTAL.

Decitrated lemon juice was prepared in the usual way. As oxidising agents were used 2:6-dibromophenolindophenol, iodine and hydrogen peroxide. The juices immediately after oxidation were placed in gas-washing-bottles, which, after hydrogen sulphide had been passed through for a suitable length of time, were sealed up for 24 hours. The hydrogen sulphide was subsequently removed by blowing oxygen-free nitrogen through the bottles.

Titration with indophenol was made with a $N/1000$ solution of this reagent, which was standardised against titanous chloride. The titrations were carried out in neutral solution.

Titration with iodine was carried out as follows. 5 cc. of the juice were mixed with 1 cc. glacial acetic acid and then 10 cc. $N/100$ iodine run in. After standing 5 minutes the excess iodine was determined with $N/100$ sodium thio-sulphate. In the case of raw juice, no acetic acid was added.

Oxidations with iodine in acid solution.

(1) *Raw juice.* 5 cc. of this juice reduced 41 cc. $N/1000$ indophenol and 5.1 cc. $N/100$ iodine.

100 cc. of the juice were mixed with 10.2 cc. $N/10$ iodine and left to stand for 30 minutes. Through the mixture, which possessed no capacity for reducing indophenol, hydrogen sulphide was bubbled for 10 minutes. The bottle was then sealed up for 24 hours, after which a vigorous stream of oxygen-free nitrogen was blown through the juice for 3 hours. At the end of the experiment, the equivalent of 5 cc. of the original juice reduced 38 cc. $N/1000$ indophenol and 4.9 cc. of $N/100$ iodine.

(2) *Decitrated lemon juice.* The juice used was such that 5 cc. reduced 27 cc. $N/1000$ indophenol and 3.4 cc. $N/100$ iodine.

100 cc. of the juice were acidified with 10 cc. of glacial acetic acid; 6.8 cc. $N/10$ iodine and 3.2 cc. of water were then added. The remainder of the procedure was essentially the same as the above.

5 cc. equivalent of the original juice at the end of the experiment reduced 28 cc. *N*/1000 indophenol and 3.8 cc. *N*/100 iodine.

In both these experiments, good agreement was observed between the reducing capacities of the original and regenerated juices.

Oxidations with 2:6-dibromophenolindophenol.

Raw juice. 5 cc. of juice originally reduced 24 cc. *N*/1000 indophenol.

50 cc. of the raw juice were brought with *N* NaOH to p_H 6.8. The requisite amount of 2:6-dibromophenolindophenol in 10 cc. of water was added and the mixture diluted to 125 cc. 100 cc. of this mixture were next treated as in the previous experiment, except that the liquid was centrifuged before removal of the hydrogen sulphide. Bubbling nitrogen for 3 hours reduced the hydrogen sulphide to the required minimum. An amount of juice equivalent to 5 cc. of the original reduced 15 cc. of *N*/1000 indophenol.

Decitrated lemon juice. 5 cc. of juice reduced 24 cc. *N*/1000 indophenol.

The requisite amount of 2:6-dibromophenolindophenol in 10 cc. of water was added to 50 cc. of juice and the mixture diluted to 75 cc. It was then treated as described above. The hydrogen sulphide was removed by bubbling nitrogen for 3 hours. An amount of the treated juice equivalent to 5 cc. of the original juice reduced 12 cc. of *N*/1000 indophenol.

In both these experiments the low figures are probably to be attributed to the presence of the reduced form of the indophenol.

Oxidations with hydrogen peroxide.

Oxidations with this reagent are much slower than with either of those used in the previous experiments. Great difficulty was experienced in removing the hydrogen sulphide from the raw juices which were used. Even after passing nitrogen for 7 hours the indophenol and iodine titrations, as well as the qualitative tests, revealed the presence of hydrogen sulphide. The following experiment, however, shows that a quantitative regeneration of the reducing capacity of the juice can be obtained after oxidation with hydrogen peroxide.

5 cc. of the juice used reduced 24 cc. of *N*/1000 indophenol and 3.4 cc. *N*/100 iodine. 13.6 cc. of *N*/10 hydrogen peroxide were run into 200 cc. of the juice, and then 6.4 cc. of water were added. After standing for 3 hours 5 cc. equivalent of the original juice reduced 5.5 cc. of *N*/1000 indophenol. The juice was treated as in the previous experiments. Nitrogen was blown through the juice for 15½ hours before the hydrogen sulphide was reduced to the required minimum. The equivalent of 5 cc. of the original juice then reduced 23 cc. of *N*/1000 indophenol and 3.4 cc. of *N*/100 iodine.

SUMMARY.

Tillmans's observation that lemon juice oxidised with indophenol, iodine or hydrogen peroxide can regain its reducing capacity when treated with hydrogen sulphide immediately after oxidation is confirmed.

My thanks are due to Dr S. S. Zilva for help and criticism and to the Medical Research Council for a whole time grant.

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CLXXV. THE VITAMIN CONTENT OF THE MANGO FRUIT.

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(Received July 3rd, 1933.)

In a preliminary investigation [Perry and Zilva, 1932], the vitamins A, C and D of three varieties of mango, namely Alphonso, Cawasji Patel and Shendrya, were assayed. It was found that the vitamin A content of the first variety was approximately that of a good butter and that the other two varieties were about half as active. The antiscorbutic activity of the three fruits showed a much greater variation. Thus whilst the Alphonso was about twice as active as lemon juice, little vitamin C was found in Shendrya, and Cawasji Patel, although not as active as Alphonso, showed nevertheless a very good vitamin C content. The presence of vitamin D on the other hand could not be established in any of the fruits in the doses tested.

In this communication, experiments are described which show that the above observations on the relative vitamin potency of the varieties mentioned were not accidental, but that they very probably hold true in general.

Guha and Chakravorty [1933] claim that the mango varieties Bombai, Langra and Fozli are rich in vitamin A but vary in their vitamin C content. Their experimental details are however not fully documented.

EXPERIMENTAL.

Treatment of experimental material. The mangoes on arrival in England were ripened in a warm, airy room if necessary. Owing to their perishable character and in order to preserve the vitamins, the fruits were then placed in a refrigerator at -20° and kept at that temperature during the tests, vitamin C being stable under these conditions [Bracewell *et al.*, 1931]. As the daily weighing out of so many small doses would have been inaccurate and unnecessarily laborious, a week's supply was weighed out for each animal and one sixth of the weekly ration administered every day except Sundays. The weekly doses were stored at -20° and thawed immediately before being administered to the experimental animals.

Methods. Vitamin A was assayed on rats by a curative growth method based on Steenbock's principle [Crawford *et al.*, 1932].

Vitamin C was tested prophylactically [Bracewell *et al.*, 1930].

The method of Chick *et al.* [1926] was used in the determination of vitamin D.

The three Poona varieties: Alphonso, Cawasji Patel and Shendrya, were despatched in two consignments, on June 11th and June 17th, 1932. The analytical data are given in Table I. These two consignments were pooled for the biological tests. Two other samples of Alphonso, of Surat and Bombay origin, were despatched on June 25th and tested separately. The analytical data

Table I. *Chemical composition of mangoes tested.*

Mangoes despatched from India, June 11th, 1932.

	Alphonso (Poona)	Shendrya (Poona)
Acidity in terms of H_2SO_4 } % of	0.28	0.16
Total sugars as glucose } pulp	14.21	16.19

Mangoes despatched from India, June 17th, 1932.

	Alphonso (Poona)	Cawasji Patel (Poona)	Shendrya (Poona)
Acidity in terms of H_2SO_4 } % of	0.23	0.29	0.11
Total sugars as glucose } pulp	13.84	16.04	20.26

Mangoes despatched from India, June 25th, 1932.

	Alphonso (Surat)	Alphonso (Bombay)
Acidity in terms of H_2SO_4 } % of	0.33	0.37
Total sugars as glucose } pulp	15.09	16.69

Table II. *Tests for vitamin A value of mango pulp.*

Alphonso (Poona)			Cawasji Patel (Poona)			Shendrya (Poona)		
Dose in g.	Number of rat, litter and sex	Change in weight during 4 weeks g.	Dose in g.	Number of rat, litter and sex	Change in weight during 4 weeks g.	Dose in g.	Number of rat, litter and sex	Change in weight during 4 weeks g.
0.05	2689 VIII σ_1	*	0.05	2672 VI σ	15	0.05	2674 VI σ_1	*
	2698 IX σ_1	34		2691 VIII σ_1	7		2686 VIII σ_1	*
	2750 XI σ_1	32		2700 IX σ_1	*		2706 X σ_1	*
	2760 XII σ_1	28		2751 XI σ_1	*		2758 XI σ_1	29
	2807 XVIII σ_1	12		2810 XVIII σ_1	*		2764 XII σ_1	19
	2834 XXI σ_1	20		2837 XXI σ_1	22		2833 XXI σ_1	*
0.1	2655 IV σ_1	64	0.1	2661 IV σ_1	*	0.1	2663 IV σ_1	4
	2664 V σ_1	43		2670 V σ_1	9		2685 VIII σ_1	3
	2688 VIII σ_1	14		2694 VIII σ_1	*		2753 XI σ_1	29
	2755 XI σ_1	40		2773 XIII σ_1	*		2772 XIII σ_1	70
	2770 XIII σ_1	45		2777 XIV σ_1	21		2794 XVI σ_1	29
	2814 XVIII σ_1	37		2813 XVIII σ_1	41		2811 XVIII σ_1	25
0.2	2648 III σ_1	46	0.2	2633 I σ_1	71	0.2	2640 I σ_1	45
	2761 XII σ_1	56		2681 VII σ_1	53		2683 VII σ_1	54
	2767 XIII σ_1	4		2695 IX σ_1	50		2762 XII σ_1	64
	2779 XIV σ_1	31		2766 XII σ_1	56		2776 XIII σ_1	43
	2792 XVI σ_1	32		2778 XIV σ_1	37		2780 XIV σ_1	*
	2815 XVIII σ_1	*		2793 XVI σ_1	*		2795 XVI σ_1	34

Positive controls. Cod-liver oil

Dose in g.	Number of rat, litter and sex	Change in weight during 4 weeks g.
0.1	2662 IV σ	70
	2679 VII σ	81
	2692 VIII σ	65
	2712 X σ	70
	2765 XII σ	76
	2768 XIII σ	93
	2809 XVIII σ	74
	2839 XXI σ	60

Negative controls

Dose	Number of rat, litter and sex	Change in weight during 4 weeks g.
No dose	2667 V σ	*
	2675 VII σ	*
	2690 VIII σ	*
	2709 X σ	*
	2759 XII σ	*
	2774 XIII σ	*
	2840 XXI σ	*

* Lost weight and died.

Table III. *Tests for vitamin A value of mango pulp.*

Alphonso (Surat)			Alphonso (Bombay)		
Dose in g.	Number of rat, litter and sex	Change in weight during 4 weeks g.	Dose in g.	Number of rat, litter and sex	Change in weight during 4 weeks g.
0.05	2784 XV	-6	0.05	2805 XVII	-19
	2798 XVII	*		2820 XIX	34
	2817 XIX	*		2826 XX	*
	2823 XX	12		2848 XXIII	*
	2858 XXIV	19		2868 XXV	20
	2872 XXVI	10		2873 XXVI	-2
0.1	2789 XV	*	0.1	2804 XVII	*
	2806 XVII	18		2822 XIX	25
	2816 XIX	*		2849 XXIII	34
	2842 XXII	46		2854 XXIII	*
	2853 XXIII	57		2874 XXVI	15
	2876 XXVI	27		2877 XXVI	16
0.2	2790 XV	77	0.2	2788 XV	46
	2801 XVII	*		2802 XVII	1
	2824 XX	22		2845 XXII	25
	2843 XXII	21		2864 XXV	35
	2850 XXIII	21		2870 XXV	35
	2867 XXV	50		2875 XXVI	-1
	2879 XXVI	33			
Positive controls. Cod-liver oil			Negative controls		
Dose in g.	Number of rat, litter and sex	Change in weight during 4 weeks g.	Dose	Number of rat, litter and sex	Change in weight during 4 weeks g.
0.1	2783 XV	72	No dose	2785 XV	*
	2799 XVII	52		2797 XVII	*
	2829 XX	47		2821 XIX	*
	2863 XXIV	73		2830 XX	*
	2869 XXV	90		2847 XXII	*
				2859 XXIV	*
				2866 XXV	*

* Lost weight and died.

Table IV. *Summary of tests for vitamin C of mango pulp.*

Dose in gm.	0.25	0.5	1	2	Approximate equivalents in vitamin C international units per g.
Alphonso (Poona)	+	++++	++++		20-30
Cawasji Patel	⊥ to +	+++	++++		15-20
Shendrya	-	-	⊥	+ to +⊥	2
Alphonso (Surat)	+++	+++⊥	++++		20-30
Alphonso (Bombay)	+⊥ to ++	++++	++++		20-30
Dose in cc.	0.5	1	1.5		
Decitrated lemon juice	+	+++⊥	++++		

for these are also given in Table I. The range of acidity (as H_2SO_4) was from 0.11 to 0.37 % and of sugar content (as glucose) from 13.84 to 20.26 %, whilst the corresponding figures for the experimental batches used in 1931, which were less ripe when picked, were from 1.06 to 3.74 % for the acid content and from traces to 8.05 % for the sugar content.

The results of the biological tests are incorporated in Tables II to IV.

Vitamin A. It will be seen from Table II that, as in the 1931 experiment, the best results were obtained with 0.2 g. of the Alphonso variety although in this case also growth was not optimum. The other two varieties did not show such high activity. From a comparison of Tables II and III it transpires that the vitamin A activity of the Alphonso variety does not seem to be affected by the place of origin.

Vitamin C. The results are summarised in Table IV. Almost complete protection was obtained with a dose of 0.5 g. of any of the Alphonso mangoes, showing the potency to be of a similar order to that of the fruit of the 1931 season. The results with the lowest dose (0.25 g.) suggest that the Alphonso (Surat) and Alphonso (Bombay) varieties are rather more potent than Alphonso (Poona). The Cawasji Patel variety is rather less potent than Alphonso, and Shendrya shows only slight activity. This observation is similar to that made on the varieties in 1931.

Vitamin D. Owing to the negative results obtained in 1931 with 0.2 g. of mango of any variety, doses of 0.6 g. of Alphonso (Poona), Cawasji Patel and Shendrya and of 1 g. of Alphonso (Surat) and Alphonso (Bombay) were tested in 1932. The results as before were all negative.

CONCLUSIONS.

In the experiments carried out on the mangoes grown in 1931, fruits were utilised which arrived in an unripe condition. It was therefore desirable in repeating this work to employ experimental material which was riper at the time of picking, in order to ascertain whether the maturity factor had any bearing on the vitamin content of the mango. No striking indications were obtained in this direction. The vitamin C content was found to be somewhat higher in 1932 than in 1931. The difference in activity is however not very great and is most probably accidental.

We wish to thank Dr S. S. Zilva for help and advice, and the Horticulturist and the Agricultural Chemist to the Government of Bombay for supplying us with the experimental material and the analytical data.

The expenses of this investigation were defrayed by the Medical Research Council out of a grant made to them by the Empire Marketing Board, to whom we should like to express our indebtedness.

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CLXXVI. VITAMIN C IN CITRUS JUICES.

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(Received May 13th, 1933.)

THE recent work of Svirbely and Szent-Györgyi [1933] has brought almost conclusive proof of the identity of vitamin C and the ascorbic acid which can be prepared from orange juice, paprika, or suprarenal glands, and it has been shown by several workers that there is a close connection between the amount of ascorbic acid as determined by titration with Tillmans's reagent, dichlorophenolindophenol, and the antiscorbutic value of the material examined.

In the experiments described in the following pages this method of titration has been applied to the examination of lemon and orange juices with the object of ascertaining the degree of natural variation in the content of ascorbic acid and the conditions which determine its preservation or disappearance in storage.

Harris and Ray [1933] give reasons for preferring to carry out this titration in acid solution (p_H 2.5) rather than in the nearly neutral solution proposed by Tillmans. They find that with fresh juice the results are practically identical but that if the juice is boiled or aerated in order to destroy the vitamin little diminution is found in the reducing power as determined by titration in neutral solution, whereas acid titration shows a much more marked falling-off.

The titrations here to be described were made in very slightly acid solution, p_H about 6. At this acidity the indicator gives a violet-blue coloration whereas in more acid solution the colour is red, and in neutral solution indigo blue. At this reaction— p_H 6—the end-point is much more satisfactory than in the acid state, the red coloration being difficult to see, while in neutral solution the reduction of the indicator takes place slowly so that the titration becomes rather tedious. At p_H 6 the indicator can be run in rapidly and the colour disappears instantaneously until near the end when it slows down, and the titration is considered to be finished when the violet-blue colour is permanent for half a minute.

The titrated liquid left exposed to the air gradually becomes dark blue from reoxidation of the indicator.

It is found that the indicator solution if made up in accordance with the suggestion of Tillmans, Hirsch and Hirsch [1932], in Sørensen's phosphate buffer solution of p_H 7 keeps better than in water and this solution was generally used for the titrations. As described by these authors the solution was standardised by titration with titanous chloride, this, in turn, being standardised against ferric ammonium sulphate.

The results are given as cc. of $N/1000$ solution of the indicator per 1 cc. of juice, and are not calculated to ascorbic acid since it is possible that other substances capable of reacting with the indicator may be present in the juice and that further work may lead to some correction for these.

Procedure. 2 cc. of juice are measured into a small beaker and diluted with about 10 cc. of water. Add 3–4 cc. of 10 % sodium acetate solution and titrate with approximately $N/1000$ solution of dichlorophenolindophenol until the violet-blue colour is persistent for half a minute. In the case of orange juice,

whose acidity is so much lower than that of the lemon, the sodium acetate should be followed by a drop of acetic acid.

Some of the results obtained with lemon juice are shown in Table I. (The fruit was obtained from various districts and squeezed in the laboratory using an ordinary glass lemon squeezer. The juice was passed through a strainer and titrated immediately.)

Table I.

Sample	cc. N/1000 indicator per 1 cc. juice	Acidity g. citric acid per 100 cc.	Sample	cc. N/1000 indicator per 1 cc. juice	Acidity g. citric acid per 100 cc.
1	9.1	6.85	12	8.45	7.0
2	10.7	7.35	13	8.1	6.65
3	10.5	7.40	14	8.25	7.07
4	8.5	6.85	15	9.05	6.95
5	9.7	7.65	16	6.25	7.35
6	8.25	6.80	17	8.00	6.65
7	10.45	7.85	18	7.5	6.80
8	10.65	7.70	19	8.6	7.25
9	9.55	7.30	20	7.2	7.05
10	8.1	7.35	21	8.0	7.65
11	7.7	7.60			

It is seen that the variations are very considerable, the lowest value being only about 60 % of the highest and the doses of juice used in biological trials may have quite different values in different cases.

It has not been possible so far to see any relation between the reducing value of the juice and its acidity or other properties. Nor is there any clear connection with the degree of ripeness of the fruit, results of similar value being found in December and in March.

Even more striking variations were found between individual lemons. Twenty lemons gathered from the same tree on March 2nd and squeezed separately gave results varying from 4.9 to 10.0.

Orange juice is more nearly uniform and the results are distinctly higher. All the samples examined have been found to lie between 10.3 and 11.8.

Effect of preservatives. It was found that lemon and orange juices without any addition of preservatives could be kept for long periods without any appreciable diminution in their reducing power. Although they ferment, and even when the surface is covered with a growth of mould, the reducing power is almost unaltered.

In the presence of preservatives this is not the case. In Table II are set out the results of an experiment in which freshly prepared lemon juice was divided into a number of portions. One was left without any addition and to the others were added the indicated quantities of various preservatives. The bottles were left at the ordinary laboratory temperature (between 16° and 22° during the period of the experiment) and titrated at intervals. In the unpreserved juice, fermentation sets in within 48 hours and is tolerably complete within a week.

The sugars were added in the form of 80 % syrups so that the juice was diluted to twice its volume. Fermentation began in the first few days and the titration value remained practically constant thereafter.

It is seen that formaldehyde has an immediately destructive effect on the reducing factor. The other preservatives do not seem to act on it directly, but in all the unfermented juices the reducing factor had practically disappeared in 30 days.

The experiments in presence of sugar were repeated with addition of sulphur dioxide or benzoic acid as preservatives (Exps. 10 and 11).

Table II.

Preservative employed	Titrations				
	Original	After			
		2 days	7 days	30 days	95 days
1. None	7.80	7.50	7.50	7.35	6.90
2. Sulphur dioxide 0.035 %	7.80	7.40	6.90	0.60	—
3. Benzoic acid 0.063 %	7.80	6.30	5.60	0.50	—
4. Sodium fluoride 0.10 %	7.80	5.80	2.20	0.30	—
5. Formic acid 0.30 %	7.80	5.60	2.00	—	—
6. Formaldehyde 0.30 %	7.80	0.50	—	—	—
7. Salicylic acid 0.05 %	7.80	6.20	3.20	0.50	—
8. Glucose 40 %	3.90	3.60	3.60	3.50	—
9. Sucrose 40 %	3.90	3.75	3.75	3.60	—
	Original	3 days	8 days	16 days	37 days
10. 40 % sucrose + SO ₂ 0.035 %	4.55	4.0	4.0	3.2	1.2
11. 40 % sucrose + benzoic acid 0.063 %	4.55	3.8	2.9	1.0	—

In this and in other experiments the juices preserved with sulphur dioxide retained their reducing power longer than the others, but even in this case it disappeared almost entirely within 30 or 40 days. The sulphurous acid itself reduces the indicator though more slowly than the reducing factor of the juice and no good end-point can be obtained in its presence. The method followed is to distil off the sulphur dioxide in a current of carbon dioxide, cool rapidly, dilute to the original volume and titrate. It was found that this procedure had no effect on the titration value of pure juice or of juice to which sulphurous acid had been quite recently added.

The experiment was tried of adding much larger quantities of sulphurous acid, but this had little effect on the rate of loss of the reducing factor.

Amount of SO₂ added 2.85 g. per litre (10 times usual addition):

Titration.	Original	7.5
"	After 12 hours	7.5
"	" 5 days	7.4
"	" 12 "	5.8
"	" 19 "	4.1
"	" 28 "	2.9
"	" 42 "	1.1

In 4 weeks the reducing factor had diminished to one-third of its original value but the amount of SO₂ still present was 1.6 g. per litre—five times as much as is usually employed as preservative.

Effect of alcohol. The addition of alcohol to juice caused a rapid loss of reducing power.

	Original titration	3 days	8 days	16 days	37 days
Juice containing 10 % of alcohol	8.55	6.9	5.0	1.1	—

With 50 % of alcohol all reducing power was lost in 5 days.

Effect of acidification.

	Original titration	18 hours	8 days	15 days
90 cc. of juice + 1 cc. hydrochloric acid, p_H 1.8	8.75	7.8	1.4	—
90 cc. of juice + 5 cc. hydrochloric acid, p_H 1.2	8.3	7.2	0.9	—

The reducing power falls off rapidly and is practically gone within a week. The acidity is too high for fermentation to take place.

Effect of traces of metals.

	Original	24 hours	96 hours	5 days	12 days	28 days
Juice + 0.001 % copper	8.0	6.5	5.6	5.6	5.6	5.6
„ + 0.003 % „	8.0	6.2	4.3	—	—	4.3
„ + 0.001 % iron	8.0	7.8	7.8	7.8	7.8	7.7
„ + 0.001 % aluminium	8.0	7.8	7.8	7.8	7.8	7.7

Iron and aluminium have no effect. Copper causes a rather rapid decrease until fermentation begins and then has no further effect.

Effect of pasteurisation. Juice contained in a series of test-tubes was heated at 65° for an hour. The tubes were stoppered with cotton-wool and tested at intervals.

Original titration	8.0
Immediately after pasteurisation				7.5
After 16 hours	7.2
„ 2 days	6.8
„ 4 „	5.1
„ 6 „	4.7
„ 8 „	3.5
„ 11 „	3.1
„ 13 „	2.2
„ 18 „	—

A steady diminution of the reducing factor occurs, leading to disappearance in 18 days. Boiling juice under a reflux condenser for several hours causes only a small decrease in reducing power. The boiled juice, if protected from fermentation, follows the same course as the pasteurised juice shown above. In this respect we do not find any important difference when the titrations are carried out in acid solution as suggested by Harris and Ray.

Titration of original juice	9.7
„ after boiling for 2 hours under reflux	9.5
„ after boiling for 2 hours under reflux (titrated at natural acidity of juice)	9.3

Effect of lemon oil. The results in presence of lemon oil are somewhat irregular and this is to be expected. The oil is only soluble to a very slight extent in the juice so that anything beyond a very small quantity forms a layer on the surface of greater or less thickness and this layer may to some extent be protective. Oil is not very efficient as an anti-fermentative, but affords considerable protection against the growth of moulds.

Experiments were made with 0.2, 0.5 and 1.0 % of oil. In all cases there was a fairly rapid diminution in reducing power in the first few days. With the smaller quantities this fall was arrested after the fifth day and the value then remained constant for several weeks at about two-thirds of the original figure. With 1 % the reducing power continued to diminish slowly and in 2 months had almost disappeared.

In experiments with juice pasteurised by heating for an hour at 65° similar results were obtained. The juice thus treated loses its reducing power at about the same rate as when treated with benzoic acid or similar preservatives. If, however, at any time ferments are allowed access to the liquid the loss is arrested and the reducing power remains nearly constant at the point it had reached before fermentation began.

The loss of reducing power while in the sterilised state is greatly retarded if precautions are taken to extract any dissolved air from the juice and to keep it thereafter out of contact with the atmosphere, and experiments are now in course to determine the duration of the reducing factor in these circumstances.

All these observations are in close agreement with the work of Williams and Corran [1930] who used the biological method and concluded that those substances which exert the strongest preservative action against gross fermentation possess the greatest destructive action on the antiscorbutic vitamin system.

It seems probable, however, that the preservatives, except formaldehyde, do not directly attack the reducing factor, but that they inhibit the action of another factor which in the untreated juice protects the vitamin from atmospheric oxidation.

This protective agency being destroyed by all the usual anti-fermentatives and by heat, and being restored if fermentation is set up in the once sterilised liquid before the reducing factor has disappeared, must be of the nature of an enzyme [*cf.* Zilva, 1928].

Orange juice. The behaviour of orange juice is, in general, similar to that of lemon juice although one or two inconsistencies have been observed which are still the subject of study.

Preservative employed	Titrations			
	Original	After 8 days	20 days	40 days
1. None	11.25	10.95	10.95	10.65
2. Sulphur dioxide 0.035 %	11.25	9.40	8.10	6.65
3. Benzoic acid 0.063 %	11.25	7.80	0.75	—
4. Sodium fluoride 0.10 %	11.25	7.70	0.60	—

In this series it is seen that the reducing power is maintained in the presence of sulphurous acid to a greater extent than was found to be the case with lemon juice, quite half of it remaining after 6 weeks.

With fluorides or benzoates on the other hand the loss of reducing power was almost complete in 3 weeks. In other experiments, however, when, after addition of benzoate, the juice was placed in a vacuum for some time in order to extract as far as possible any air, dissolved or adhering in minute bubbles to the floating pulp, the diminution of reducing power took place much more slowly. After 3 weeks the titration was 6.7, and after 6 weeks 6.25. The loss continued slowly and final disappearance of the reducing power required 4 months.

Further experiments were made with juice from blood oranges squeezed in April and therefore much riper than the fruit previously used. After addition of the usual proportion of benzoic acid the juice was stirred gently *in vacuo*, and was then preserved in ordinary corked bottles. To one of these was added sufficient citric acid (5 %) to bring its acidity up to that of average lemon juice.

Treatment	Titrations			
	Original	After 10 days	15 days	24 days
1. None	11.25	10.0	10.0	9.8
2. Benzoic acid 0.063 %	11.25	8.1	6.5	6.1
3. Do.	11.25	8.0	6.5	5.4
4. Do. + 5 % citric acid	11.25	5.0	3.1	0.4

The acidified juice behaves like lemon juice. The unacidified juice loses its reducing power at a slower rate.

Acidification with hydrochloric acid has, as with lemon juice, the effect of

accelerating the loss of reducing power provided the amount added is sufficient to arrest fermentation.

	Original titration	5 days	8 days	12 days	18 days
1. 90 cc. of juice + 1 cc. hydrochloric acid, p_H 2.1	10.3	9.5	9.5	9.3	9.3
2. 90 cc. of juice + 4 cc. hydrochloric acid, p_H 1.2	10.1	8.5	7.0	4.0	2.6

In No. 1 the acidity was not sufficient to prevent fermentation and the reducing power, in consequence, remained nearly constant.

The p_H of the orange juice before addition of acid was 3.6. That of lemon juice is about 2.1 to 2.3.

Fractionation. The fractionation of the reducing or antiscorbutic factor in lemon juice when this is treated with lead acetate at various p_H values has been the subject of considerable discussion. Zilva [1932] is of opinion that there is no definite relation between the reducing powers of such fractions and their antiscorbutic values, while at the same time there is no clear-cut separation of vitamin in the different precipitates. These in fact, voluminous and difficult to wash, are of the type most likely to retain small quantities of other substances, and any extended manipulation of them is to be avoided since the reducing factor is here in a highly unstable state. In the course of a few hours the solution of one of these precipitates loses a large part of its reducing power.

It is not clear from Zilva's paper what proportion of the total antiscorbutic factor he found to be contained in the precipitate at p_H 5.4. From the observations of Tillmans, Hirsch and collaborators [1932] it seems that about one-fifth of the original reducing power was precipitated at this point.

Working with small quantities of juice which permit rapid manipulation we have found about 10 % of the reducing power to be removed in this precipitate.

A second precipitate was obtained by adding ammonia to the first filtrate until the p_H reached 8.0 and the filtrate from this had no reducing power. The second precipitate (p_H 5.4–8.0), redissolved in acetic acid, was found to contain over 60 % of the reducing factor originally present.

Taken 250 cc. of lemon juice of which 1 cc. requires 10.5 cc. *N*/1000 indicator solution.

- | | | | | | |
|----------------------|-----|-----|-----|-----|----------|
| Total reducing power | ... | ... | ... | ... | 2625 cc. |
|----------------------|-----|-----|-----|-----|----------|
- Added 13.5 g. calcium carbonate, stirring well. Heated to 85°, filtered and washed. Filtrate 500 cc. of which 1 cc. requires 5.15 cc. indicator.

Total reducing power of filtrate	2575 cc.
p_H of filtrate	5.4.				
 - Added 10 g. lead acetate, filtered and washed. Filtrate 800 cc. of which 1 cc. requires 2.93 cc. indicator.

Total reducing power of filtrate	2340 cc.
Loss between 1 and 2	235 cc.
<i>i.e.</i> 9 % of total present.					
 - Added ammonia to p_H 8.0; filtered and washed. Filtrate had no reducing power.
 - Precipitate redissolved in dilute acetic acid. Volume of solution 170 cc. 1 cc. required 9.5 cc. of the indicator solution.

Total reducing power	1615 cc.
<i>i.e.</i> 61.5 % of the reducing power originally present.					

The precipitate was decomposed with hydrogen sulphide, filtered, washed, the hydrogen sulphide expelled in a current of carbon dioxide and the liquid concentrated in vacuum to 60 cc. without further loss of reducing power.

In another experiment with 500 cc. of juice nearly 70 % of the original reducing power was obtained in the concentrate but with larger volumes of juice it has not, so far, been possible to get more than about 40 %, the loss taking place during the formation and manipulation of the second lead precipitate.

Commercial juices. Mr F. K. Donovan has been kind enough to make some titrations of commercial juices and of juices pressed by himself in London for comparison with our results.

Lemon juice freshly pressed in London gave titrations of from 7.7 to 9.45, results well within the range we have found in Sicily. Commercial juices, generally preserved with sulphurous acid, and the syrups made from them, gave, as would be expected from the foregoing observations, much lower results. The highest titration found was 4.7 and in other cases hardly any reducing power remained.

Imported lime juice, preserved by its oil only, gave 2.2 and 1.35. When freshly pressed from limes the figures found were 4.85 and 4.9. Hassan and Basili [1932] have published experiments with Egyptian limes which led them to the conclusion that fresh juice is active but loses its activity more rapidly than lemon juice, and that the loss begins in the fruit itself during ripening or storage. The limes from which juice was pressed in London were of unknown history and probably juice from newly gathered fruit might have considerably higher values.

Imported West Indian grape fruit juice gave titrations between 4 and 5 and freshly pressed juice was not much higher. Sicilian grape fruit pressed shortly after gathering, however, gave a result comparable with that of lemon juice—7.5.

Experiments are now in course in which the reducing power of juices preserved in various ways, including juices sterilised by the Matzka process, will be followed during a period of some months and will be compared with the results of biological trials carried out with the same material.

It is hoped that the results may be the subject of a further communication.

SUMMARY.

1. The method of titration with dichlorophenolindophenol has been applied to the examination of a number of samples of lemon and orange juices, both freshly prepared and preserved in various conditions.

2. It has been found:

(a) That the reducing power of fresh lemon juice is subject to considerable variation, the lowest samples examined having only 60 % of the reducing power of the highest.

(b) That the reducing power of orange juice is more constant and rather higher than that of lemon juice.

(c) That the reducing power of both juices does not diminish much in storage in the absence of preservatives, but that the use of any preservative which is efficient in preventing fermentation is followed by the gradual diminution of the reducing power which totally disappears in, at most, a few weeks.

(d) That the same result is brought about by strong acidification, pasteurisation or boiling.

3. It is concluded that in untreated juice the reducing factor is protected from atmospheric oxidation by the action of an enzyme, and that when this action is inhibited by any of the usual means the reducing power is rapidly lost.

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CLXXVII. THE VITAMIN D ACTIVITY OF BUTTER.

I. A CHEMICAL DIFFERENTIATION OF THE ANTI-RACHITIC FACTOR OF AUTUMN AND WINTER BUTTER FROM IRRADIATED ERGOSTEROL AND THE VITAMIN D OF COD-LIVER OIL¹.

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(Received July 1st, 1933.)

IN the course of a study of the vitamin content of typical English milk carried out in the National Institute for Research in Dairying it has been our custom to estimate vitamin D in butter in winter, summer and autumn by curative and protective experiments on rats.

As is well known, the vitamin D content of butter is at its lowest during the winter months. In 1931-32 we found that 1.5 g. of our winter butter fed daily over a period of 10 days to rachitic rats did not suffice to bring about even a partial cure of the rachitic condition, that is, we were unable to obtain even a faint "line" with this amount in the curative line test. In our technique the butter-fat is administered to the experimental animals by pipette separately from the diet, and we find it practically impossible to give to the rats more than 1.5 g. (or almost 2 cc.) of butter daily.

When planning the curative experiments for the next winter period (1932-33) we decided to concentrate the antirachitic factor of butter by saponification and to feed to rats the non-saponifiable residue of larger quantities of butter, 3 and 6 g. daily. In order to test the feasibility of such a method we decided to rehearse it in the preceding autumn and to compare, in protective experiments, the antirachitic effects of graded amounts of autumn butter with those of equivalent amounts of the non-saponifiable residue from this butter. The protective technique was chosen in preference to curative tests because, when equal numbers of experimental animals are used, it is, in our opinion, more accurate than the latter and therefore more likely to show any possible defects in our method.

EXPERIMENTAL.

Experiments carried out in autumn 1932.

Method of saponification of butter. The butter used in this study was obtained from a herd of shorthorns belonging to this Institute. The care and management of this herd are typical of the agricultural practice in the south of England [Golding *et al.*, 1932]. The butter was churned twice weekly in the Institute's dairy from fresh cream separated from mixed samples of evening's and morning's

¹ Read before the Biochemical Society, May 6th, 1933 [Kon and Booth, 1933].

milk blended in the proportion of 2:3, and was unsalted. It was melted at a temperature not exceeding 80° practically immediately after delivery from the dairy, and the rendered fat, after filtration, was stored in the ice-chest until required. The saponification was carried out as follows: to 50 g. of butter-fat 100 cc. of absolute ethyl alcohol were added, followed by 22 cc. of a saturated aqueous solution of KOH (80 g. A.R. dissolved in 54 cc. of water). The mixture was boiled on the water-bath for 1 hour, 400 cc. of water were added and the non-saponifiable matter was extracted by shaking with three 250 cc. portions of "anaesthetic" ether. The ethereal extract was washed twice with 200 cc. of water, dried over anhydrous Na_2SO_4 , the bulk of the ether driven off on the water-bath and the residue taken to dryness *in vacuo*. The non-saponifiable residue, usually amounting to about 200–250 mg., was, after weighing, dissolved in olive oil to a total weight of 5 g. This was done by dissolving the non-saponifiable residue in a small amount of ether, adding the requisite amount of olive oil and driving off the solvent *in vacuo*. In this way 1 g. of the olive oil solution contained the non-saponifiable residue from 10 g. of butter. For feeding, the oily solution was distributed, by means of calibrated pipettes, into small porcelain dishes. The butter was saponified twice weekly, 10 samples churned between October 25th and December 3rd, 1932, being used altogether for the autumn experiment. Of these the first six were distributed for feeding as described above. In the case of the last four the procedure was slightly modified, in that, after the non-saponifiable residue had been incorporated in olive oil, the solution instead of being distributed as such, was dissolved in 50 cc. of A.R. benzene. The benzene solution was then distributed into porcelain dishes from a burette. The solvent was evaporated before a fan and the last portions removed in a vacuum desiccator. The raw butter used for feeding was always from the same sample as that used for saponification.

Animal tests. For the feeding test young rats weighing 55–65 g. were used. They were left with their mothers until they reached that weight (generally between the 21st and 25th day of life). They were then randomised, placed on the 2965 rachitogenic diet of Steenbock, and the various substances to be tested for their antirachitic potency were given from the start separately from the diet for a period of 5 weeks, six times a week. At the end of the experimental period the rats were killed by coal gas, and the antirachitic potency of the substances tested was assessed by estimating the ash content of dry defatted femora and humeri and also by the appearance of sections of radii and ulnae stained with silver nitrate. Four rats were used for every substance or level tested.

In Table I we give the ash percentages obtained for several levels of autumn butter and for equivalent amounts of the non-saponifiable residue from this butter, while Plate III, fig. 1, gives photographs of stained sections of radii and ulnae of rats having received the highest level of butter and non-saponifiable residue, taken by the method of Dyer [1931] slightly modified¹.

It is obvious both from the table giving the percentages of ash and from the appearance of sections of radii and ulnae that, as a result of saponification, the antirachitic potency of butter had dropped very considerably, the non-saponifiable residue of 1½ g. of butter producing an effect only slightly superior to that given by 0.2 g. of raw butter.

It appears therefore that while these results were quite unsatisfactory so far as the elaboration of a method for the testing of butters of low antirachitic

¹ The bones, mounted according to Dyer's method, were photographed under ½ watt electric illumination, a Wratten K. 3 filter being used and the Ilford panchromatic plates being developed for only 3½–4 mins. in metol-quinol.

Table I. *Autumn 1932. Prophylactic experiment. Antirachitic effect of butter and of non-saponifiable residue from butter. Percentage of ash in dry defatted femora and humeri.*

The figures for % ash are averages of duplicate determinations on groups of 4 rats.

Negative controls 29.2 %.			
0.1 g. butter	31.0 %		
0.2 g. "	33.6	N.S.R. from 0.2 g. butter	31.4 %
0.5 g. "	40.2	" 0.5 g. "	31.4
1.0 g. "	50.0	" 1.0 g. "	33.7
1.5 g. "	51.8	" 1.5 g. "	34.5

Positive controls 52.4 %.

potency is concerned, they were highly valuable in yielding the unexpected observation that the antirachitic factor of butter does not share with other antirachitic substances their well-known resistance to saponification.

Experiments carried out in winter 1933.

In the following winter period three series of experiments were carried out. In the first series the antirachitic potency of winter butter was compared at several levels in protective experiments with that of the non-saponifiable residue of the same butter, prepared by two different methods. A mixture of irradiated ergosterol (the International Standard of vitamin D) and of the same butter was also subjected to saponification and the antirachitic potency after saponification compared with that of an equivalent dosage of the International Standard of vitamin D (0.4 unit daily).

In the second series two samples of cod-liver oil and the International Standard of vitamin D were saponified both alone and mixed with butter and the antirachitic potency of the non-saponifiable residues was compared in curative tests with that of equivalent amounts of the original substances.

In the third series, the effect of saponification on the antirachitic potency of cod-liver oil and of the International Standard of vitamin D (either alone or mixed with butter) was judged in protective experiments.

First series of winter experiments. As already mentioned butter for these experiments was saponified by two methods, one being the method previously described. In the other, instead of boiling the butter for 1 hour with alcoholic potash we brought the mixture to a temperature of 60–70° for 2 minutes only, saponification being completed under these conditions. As previously, the butter was saponified twice weekly in 50 g. lots, or, in the case of butter saponified for 1 hour in 25 g. lots. The further treatment was as previously described. For feeding, the non-saponifiable residue was dissolved in "anaesthetic" ether and transferred to a volumetric flask containing olive oil to the extent of 10 % by weight of the butter originally taken for saponification. The ethereal solution was made up to contain the equivalent of 1 g. of butter in 1 cc. It was then distributed for feeding into small porcelain dishes from a special burette for volatile liquids. Ether was used in preference to benzene because of the relatively greater ease with which the former solvent may be removed. The butter for this experiment was churned between February 10th and March 14th, 1933.

For saponification the International Standard of vitamin D (VD. 5 issued September 30th, 1932) was mixed with winter butter in the proportion of 1 unit of the Standard to 0.25 g. of the mixture, *e.g.* 76 mg. of the Standard were mixed with 18.924 g. of butter. The mixture was then saponified for 1 hour and the

non-saponifiable residue extracted by a method exactly similar to that used for the extraction of the non-saponifiable residue in the first experiment mentioned. For feeding, the non-saponifiable residue was dissolved in ether and distributed exactly as described in the case of winter butter.

The ash percentages obtained in this experiment are given in Table II, while in Plate III, figs. 2*a* and 2*b* are given photographs of stained sections of radii and ulnae of the experimental animals.

Table II. *Winter 1933. First prophylactic experiment. Antirachitic effect of butter, of the non-saponifiable residue from butter and of the International Standard of vitamin D before and after saponification (mixed with butter). Percentage of ash in dry defatted femora and humeri.*

The figures for % ash are averages of duplicate determinations on groups of 4 rats.

Negative controls 32.2 %.					
0.25 g. butter	37.1 %	N.S.R. from 0.25 g. butter	35.3 %		
0.5 g. "	46.0	" 0.5 g. "	36.3		
1.0 g. "	50.7	" 1.0 g. "	39.7		
1.5 g. "	53.2	" 1.5 g. "	39.2		
N.S.R. from 1.5 g. butter saponified for 1 hour			41.2 %		
0.2 unit of International vitamin D standard			46.9		
0.4 unit of International vitamin D standard			50.2		
0.4 unit of International vitamin D standard mixed with butter and saponified ...			50.0		

Positive controls 50.7 %.

It will be seen that, as far as butter is concerned, the results are very similar to those obtained in the preceding autumn.

Here again the antirachitic effect of the non-saponifiable residue from 1.5 g. of butter is only slightly superior to that given by the lowest level of raw butter, and the milder saponification does not prevent the loss of potency to any extent.

On the other hand the antirachitic activity of the International Standard of vitamin D has survived unimpaired a saponification in the presence of butter.

Second series of winter experiments. As this series overlapped the first series, the same butter, saponified butter and saponified mixture of butter and International Standard of vitamin D were used in the earlier part of this experiment (butters churned between March 10th and March 14th, 1933) as in the first series. Following on this, butters churned between March 14th and 21st were tested. In addition, two samples of cod-liver oil were used, one kindly given us by Dr K. H. Coward and assessed by her as containing 250-300 International units of vitamin D per g. For the other, one of us (S. K. K.) is indebted to Dr A. D. Holmes of the E.L. Patch Co. in Boston who sent him several samples of this oil in 1929. At that time its potency was given by Dr Holmes as in excess of 125 units (as defined in the E.L. Patch Laboratories and similar to the Steenbock unit) per g.

The cod-liver oils were tested, either as received, or after saponification, either alone or mixed with butter. The raw cod-liver oils were diluted with olive oil so as to contain the requisite amount in 20 mm.³ and were fed from pipettes made in accordance with the specifications of Memorandum 10 (A) of the Dept. of Biological Standards of the National Institute for Medical Research. Dr Coward's cod-liver oil (No. 1) was fed at a level of 2 mg. daily, while of Dr Holmes's oil (No. 2) 8 mg. were given daily. The cod-liver oils were saponified for 1 hour under precisely the same conditions as previously employed for the

butter. Two saponifications of each of the cod-liver oils were carried out in the course of the 10-day feeding period. In view of the much higher activity of cod-liver oil the amounts of non-saponifiable residue fed were much smaller than in the case of butter, and for this reason the concentrates from cod-liver oil were mixed with a relatively larger volume of olive oil and likewise with a relatively larger amount of ether. Otherwise they were distributed in porcelain dishes in exactly the same way as in the case of butter.

As already mentioned, the saponified mixture of butter and irradiated ergosterol was obtained from samples used in the first series of winter experiments.

The International Standard of vitamin D alone was saponified for 1 hour under conditions similar to those described above, the only difference being that, owing to the small quantity of the substance used (1 g.), relatively more ether was used for the extraction of the non-saponifiable residue from the soap solution in order to avoid the increased losses which might occur when such small quantities were handled. Only one saponification was carried out and the olive oil solution of the non-saponifiable residue was stored in the porcelain feeding dishes in the ice-chest.

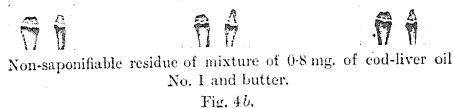
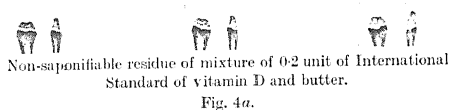
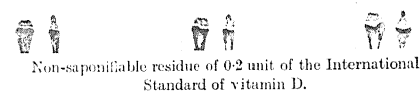
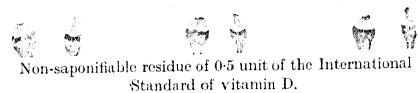
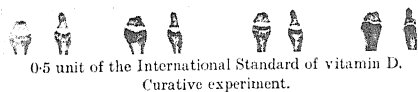
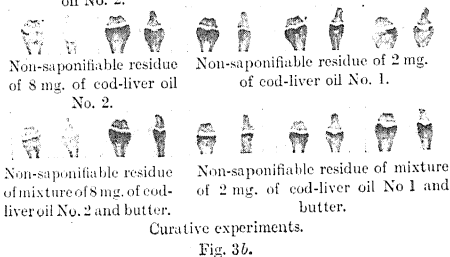
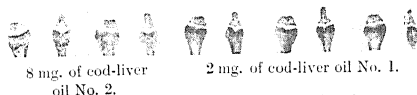
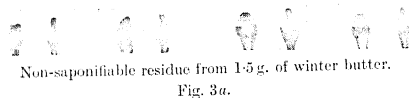
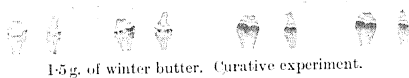
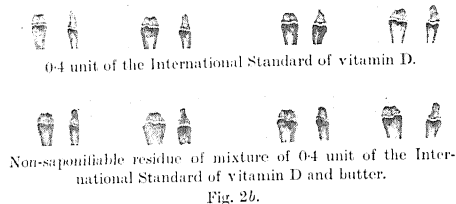
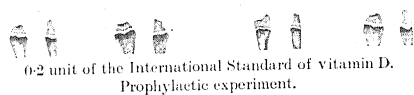
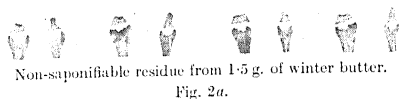
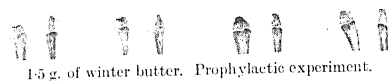
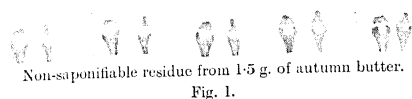
All these substances were tested on rats in curative tests, *i.e.* they were fed during the course of 10 days to rats previously rendered rachitic by being kept for 21 to 23 days on the 2965 diet of Steenbock. In Plate III, figs. 3 *a*, 3 *b* and 3 *c*, are given photographs of line tests carried out on these rats.

It will be seen that, while the two samples of cod-liver oil and the International Standard of vitamin D have retained their antirachitic potency after saponification, whether saponified alone or in the presence of butter, winter butter has again lost at least a large part of its original potency. Incidentally the butter was more potent this year than in the preceding winter and gave a definite line at the 1.5 g. level.

Third series of winter experiments. In the third series of experiments the International Standard of vitamin D (V.D. 6, distributed March 31st, 1933) was fed at 3 levels of 0.1, 0.15 and 0.2 unit daily and its antirachitic potency compared in protective experiments with that of the saponified Standard fed at the 0.2 unit level and with that of a saponified mixture of the Standard with winter butter also fed at the 0.2 unit level.

The Standard was saponified as previously described. The mixture of butter and Standard was made in the proportion of one part of the International Standard to 100 parts of the mixture; it was saponified in the usual way. At first the non-saponifiable residues were dissolved in ether and distributed as previously described. Later (10 days after the commencement of the experiment) the method was modified in the following way. The non-saponifiable residues were dissolved in a small quantity of ether and mixed with olive oil in such a way that the requisite amount of the residue was present in 20 mm.³ of the solution after evaporation of the ether. These olive oil solutions were then fed by means of vitamin D pipettes. The bulk was kept in the ice-chest, and the methods suggested by the Department of Biological Standards of the National Institute for Medical Research for the handling of the International Standard of vitamin D and of its dilutions were rigidly adhered to.

Dr Coward's cod-liver oil was fed as such after dilution with olive oil (0.8 mg. of cod-liver oil daily) and was also fed after being saponified either alone or mixed with butter in the proportion of 2.5 g. of oil to 97.5 g. of butter. These non-saponifiable residues were fed in exactly the same way as those of the International Standard of vitamin D.



For testing these substances 3 rats were used for each level (instead of the usual 4) and only 2 were available for the 0.1 unit level of the International Standard.

The ash percentages are given in Table III while photographs of stained sections of the wrist bones will be found on Plate III, figs. 4 *a* and 4 *b*. It is

Table III. *Winter 1933. Second prophylactic experiment. Antirachitic effect of cod-liver oil and of the International Standard of vitamin D before or after saponification (alone or mixed with butter). Percentage of ash in dry defatted femora and humeri.*

Ash % given as averages of duplicate determinations on groups of 3 rats (2 in the case of the 0.1 unit level of the International Standard).

Negative controls 32.7 %.

0.1 unit of International vitamin D standard	44.9 %
0.15 unit of International vitamin D standard	48.8
0.2 unit of International vitamin D standard	47.9
0.2 unit of International vitamin D standard after saponification	45.5
0.2 unit of International vitamin D standard mixed with butter and saponified	46.0
0.8 mg. cod-liver oil	48.7
0.8 mg. cod-liver oil after saponification	49.4
0.8 mg. cod-liver oil mixed with butter and saponified	49.7

evident that, under our experimental conditions, the antirachitic principle (or principles) of cod-liver oil and of the International Standard of vitamin D is not affected by saponification in the presence or absence of butter.

DISCUSSION.

When the results of several independent series of experiments described in the experimental part of the present paper are compared and reviewed as a whole, the following general conclusion may, in our opinion, be safely drawn, namely, that the antirachitic agent, whatever its nature, present in winter and autumn butters can, in experiments on rats, be chemically differentiated from irradiated ergosterol as present in the International Standard of vitamin D and from vitamin D *sensu stricto* as it is present in cod-liver oil. Under our experimental conditions we are unable to concentrate in the non-saponifiable residue the antirachitic potency of butter, whereas, under exactly similar conditions, we are able to concentrate in the same fraction practically the whole of the antirachitic activity of the two other antirachitic agents, thus simply confirming in the latter case, for our experimental conditions, the experience of countless other workers. Whether we are dealing here with a destruction of the antirachitic factor of butter or whether it only finds its way into a different fraction at some stage during the course of the preparation of the non-saponifiable residue we are at present unable to say. Experiments on this point are now in progress. Whatever the nature of the change it seems to be brought about readily in the course of saponification as evidenced by our failure to obtain any better concentration of the antirachitic factor in the non-saponifiable residue by using a milder method of saponification.

From our experiments, in which cod-liver oil and irradiated ergosterol were saponified in the presence of butter, we conclude that butter does not contain a specific destructive substance alongside the classic vitamin D, as the presence of butter does not affect the stability to saponification of other antirachitic agents. On the other hand it might be conceived that a specific adjuvant is

present in butter and enhances greatly the pharmacological action of vitamin D *sensu stricto*. While such a possibility seems to us very remote we are not prepared to rule it out without experimental evidence. This we are seeking at present in experiments designed for the purpose.

The discovery of the antirachitic activation of foodstuffs by ultra-violet irradiation and the subsequent work showing that ergosterol was apparently specific in its capacity for activation by this means have led many students of nutrition to believe that only one antirachitic substance exists and to reduce, therefore, all manifestations of antirachitic activity to the common denominator of irradiated ergosterol. Recent evidence has extensively undermined this view, in fact has rendered it untenable. The work of Hess [Hess and Supplee, 1930; Hess, Weinstock and Rivkin, 1930] and of Steenbock *et al.* [1932], to mention only two of the numerous investigators in this field, has shown beyond reasonable doubt that the biological activity of fish-liver oils is, for human beings and birds (chicken), rat-unit for rat-unit, much higher than that of irradiated ergosterol.

Our experiments on butter point to a chemical difference between winter and autumn butter on the one hand and irradiated ergosterol and cod-liver oil on the other as far as their vitamin D activities are concerned. If we accept a difference as existing between the two latter factors, and this we have no reason to deny, it seems to us that the existence of at least a trinity of antirachitic agents is now supported by a respectable weight of evidence.

After having failed to concentrate the vitamin D activity of butter in its non-saponifiable fraction we searched the literature in order to see whether findings of a similar nature had not been recorded elsewhere. Two observations have attracted our attention. In one of them, Zucker and Barnett [1922-23] mention their inability to concentrate the antirachitic activity of plant tissues and of butter by extraction with 95 % alcohol and subsequent saponification of the extract, while this method was successful when applied to cod-liver oil. We are at present repeating Zucker's experiments in order to verify whether and at what stage in his procedure the butter loses its antirachitic potency.

The second paper is by Supplee *et al.* [1931]. These authors, in investigating the antirachitic activity of irradiated milk, extracted the non-saponifiable fraction of milk-fat derived from dry milk or from butter but were unable to activate it antirachitically by ultra-violet irradiation as judged by experiments on rats and chickens, whereas they were able to render equivalent amounts of original natural milk strongly antirachitic under the same conditions.

We wish it to be clearly understood that our results, both with regard to the antirachitic potency of butter and to our inability to concentrate this potency in the non-saponifiable residue, were obtained in experiments on rats, and that our suggestions and conclusions apply to this animal only. The pathological picture brought about in the rat by deprivation of vitamin D combined with a marked alteration in the calcium : phosphorus ratio is by no means identical with rickets in the human being or with similar conditions in other species resulting from the simple withdrawal of vitamin D even in the presence of a correct mineral balance. That caution is necessary in transferring to the human being results obtained on the rat has been sufficiently emphasised by the recent work on the comparative antirachitic potencies of irradiated ergosterol and cod-liver oil.

Numerous experiments in various countries have proved beyond reasonable doubt that butter is antirachitically active when tested on rats. Its value as an antirachitic agent for other species is, however, still very debatable and is yet subject to much controversy.

We are investigating at present the effect of saponification on the antirachitic factor of summer butter and also of butters rendered highly antirachitic either by direct irradiation with ultra-violet light or by the feeding to cows of irradiated yeast or of cod-liver oil. Such butters are sufficiently antirachitic to enable us to test their potency on other species, and we would like to postpone further discussion until these results are available.

SUMMARY.

1. Autumn and winter butters, either saponified in the usual way by boiling for 1 hour on the water-bath with alcoholic KOH, or by heating with alkali for 2 minutes only, lose a large part (over 80 %) of their antirachitic potency as estimated by prophylactic experiments on rats.

2. Under exactly similar conditions, irradiated ergosterol (the International Standard of vitamin D) or cod-liver oil could be subjected to saponification either alone or mixed with butter without loss of potency.

3. The fact that the stability to saponification of the antirachitic factor of cod-liver oil and of irradiated ergosterol is not adversely influenced by the presence of butter speaks against the existence in butter of a specific destructive factor and in favour of a true chemical difference between the antirachitic factor of butter and those of cod-liver oil and irradiated ergosterol.

Our best thanks are due to Miss D. V. Dearden for the churning of the butters and to N. Gruber for much work involved in the preparation of the photographs.

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CLXXVIII. AVIAN POLYNEURITIS. FURTHER STUDIES ON THE ACTION OF VITAMIN B₁ CONCENTRATES *IN VITRO*.

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(Received June 6th, 1933.)

THE evidence from recent work has strongly suggested that vitamin B₁ is concerned with the oxidative removal of lactic acid in the brain of the pigeon. Kinnersley and Peters [1929; 1930] showed that the polyneuritis of vitamin B₁ deficiency was intimately associated with a localised accumulation of lactic acid in the brain. Gavrilescu and Peters [1931] found that the oxygen uptake of minced brain from polyneuritic birds was lower than normal in the presence of glucose, and that the addition of a vitamin B₁ concentrate *in vitro* was capable of effecting a partial reparation of this defect. More recently it has been shown [Gavrilescu *et al.*, 1932] that in the presence of lactate a similar defect in the oxidations of minced polyneuritic brain can be demonstrated, which is capable of being largely repaired by the addition of very small amounts of a vitamin B₁ concentrate *in vitro*². No significant defect in the oxygen uptake of the brain was found in the absence of added substrates or in the presence of succinate. Experiments on birds recovering from polyneuritis after dosing with vitamin B₁ concentrate [Meiklejohn *et al.*, 1932] showed that there was an improvement in the oxidative behaviour of the minced brain with lactate, corresponding to the disappearance of the nervous symptoms, and with this improvement the effect of added vitamin B₁ concentrate *in vitro* diminished. It was concluded that the vitamin B₁ concentrates contain a substance capable of repairing the same defect both in the living bird and in the isolated brain, and that this defect is in the system responsible for the increased oxygen uptake of the isolated brain in the presence of added lactate. The evidence strongly suggests that the substance in the concentrates is the same chemical entity whose absence is also the cause of the symptoms of polyneuritis, that is, vitamin B₁ itself³.

The simplest hypothesis in accord with the facts was that the defect in vitamin B₁-deficient brain lay in the oxidative removal of lactate. If this were the case the addition of vitamin B₁ concentrate, in restoring to normal the lowered oxygen uptake of the deficient brain *in vitro*, should result in an increase in the removal of lactate. This has been made the subject of an investigation, the results of which are here described. The surprising result has been obtained that there is no apparent removal of lactate corresponding to the increased oxygen uptake induced by the addition of vitamin B₁ concentrate.

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² It must be emphasised that this effect is catalytic. In the experiments described in the present paper 16 γ of solid matter in the B₁ concentrate added is sufficient to cause an extra uptake of about 0.2 cc. or 300 γ of oxygen.

³ For further evidence of identity see Passmore *et al.* [1933].

METHOD.

The principle adopted has been to compare the oxygen uptakes of two equal portions of a homogeneous mince of avitaminous cerebrum, with and without the addition of vitamin B₁ concentrate, in the presence of similar amounts of added lactate, and to estimate afterwards the amount of lactate left by each.

The technique employed was arranged to differ as little as possible from that of previous experiments [Gavrilescu *et al.*, 1932]. The birds, in head-retraction, were guillotined in the usual manner. The cerebrums only were removed and finely minced. The mince (wet weight, 0.6 to 0.8 g.) was divided into two approximately equal portions by weighing on a rough balance. Each half was then divided between two previously weighed Barcroft-Dixon bottles of usual type, each containing exactly 3 cc. of the same solution of sodium *d*-lactate¹ in phosphate-buffered Ringer of p_H 7.4, made up as described by Gavrilescu and Peters [1931, 1]. The concentration of the lactate solution was approximately 0.006 *M* in all experiments. The exact weight of tissue in each bottle was determined by a second weighing. Glass crushers were added to break up the mince, and to each of the bottles containing one-half of the mince was added 0.1 cc. of vitamin B₁ concentrate, containing approximately 1/10th pigeon day dose and 8% solid matter. To each of the bottles containing the other half of the mince was added 0.1 cc. of a control solution; this, and the vitamin solution were prepared as previously described [Gavrilescu and Peters, 1931, 1]. The Barcroft apparatus were set up with the usual provision for carbon dioxide absorption and were filled with oxygen as in previous experiments.

The oxygen uptake was measured over a period of 3 hours at 38°. In every case each apparatus was levelled-off once in the course of the experiment. After the last reading the bottles were detached and their contents treated as described below.

Discussion of method.

The method of dividing the minced tissue into two halves and comparing the behaviour of each requires that the mince shall be of a homogeneous nature. For this reason only the cerebrums have been used. On account of the small size of the pigeon's brain it has been necessary to utilise the whole of the minced cerebrum to obtain a sufficient difference in the oxygen uptake between the control and vitamin-treated samples of the mince. Unfortunately this has made it impossible to perform any further estimations on the same tissue other than the simple comparison here described.

d-Lactate has been used to reduce the total amount of lactate present to the lowest limit compatible with a reasonable oxygen uptake. For the same reason each half of the mince has been divided between only two Barcroft bottles. From this point of view it would have been more convenient to include the whole of each half of the mince in only one bottle; but dividing the tissue has two advantages, it reduces the amount of tissue in each bottle to a safe limit (100 to 200 mg.), and provides a check on the oxygen uptakes by determining each in duplicate.

¹ The sodium *d*-lactate used was freshly prepared before each experiment from a recrystallised specimen of zinc sarcocollactate by the method of Meyerhof and Lohmann [1926]. The zinc sarcocollactate was prepared, with the kind assistance of Mr R. B. Fisher, from horseflesh by a modification of Fletcher and Hopkins's method [1907]. The recrystallised specimen was dried to constant weight at 120° and stored over CaCl₂ in a vacuum desiccator. A weighed sample gave a zinc oxide yield of 99.5 % of the theoretical.

In practice it has not been found possible to divide each half of the mince into two exactly equal samples, but the oxygen uptake of each sample has always been found to be proportional to its weight, both in the control and vitamin-treated samples [see Dixon and Elliott, 1930]. The oxygen uptake per g. of tissue has been calculated from the uptake of each sample. In ten cases, from the experiments quoted in this paper, the difference between these figures from two samples of the same half of the mince has been less than 5 %, and in the remaining four less than 10 %. This is an indication of the homogeneous nature of the mince.

The following are the results of three experiments in illustration of this. They have been especially selected for the inequality of tissue between the two samples. In most experiments the division has been more equal.

Exp. No.	Wet weight of tissue mg.	Avitaminous cerebrum	
		Oxygen uptake, per g. tissue in 3 hours, in the presence of 0.006 <i>M</i> sodium <i>D</i> -lactate, cc.	
6	108	3.35	
	207	3.38	
8	206	2.71	
	147	2.69	
10	127	2.96	
	209	2.96	

In theory at least the uneven division of each half of the mince requires that both the oxygen uptake and the lactate removal of each separate sample shall be directly proportional to its mass, if the method is to be acceptable. With regard to the oxygen uptake this is shown to be the case. But the problem of lactate removal must be considered. A large lactate removal, not accompanied by an uptake of oxygen and not proportional to the amount of tissue present, might theoretically effect the validity of the results. In practice, however, the uneven division of the tissue is roughly of the same order for each of the halves of the mince whose lactate removal is compared. This should largely compensate for the error introduced by such an improbable contingency.

The calibration constants of the Barcroft apparatus, for converting scale divisions into mm.³ of gas absorbed, were determined under the exact conditions of the experiments as in previous work [Gavrilescu *et al.*, 1932]. The constants so determined have a probable error of less than ± 1 %. This was confirmed by redetermining the constant of each apparatus during the course of the research.

Subsequent treatment for the estimation of lactate.

Two methods have been employed for the precipitation of the proteins. At first trichloroacetic acid was used as a precipitant. Since it seemed possible that the results might be affected by the nature of the reagent employed, some further experiments were performed using Schenk's method, as described by Lehnartz [1928]¹.

¹ In particular reference should be made to the claim of Lehnartz that the presence of trichloroacetic acid interferes with the estimation of lactate solutions and produces variable yields. This applies to a distillation method of lactate analysis. This has not been confirmed in this research in which the method of Friedemann and Kendall [1929] has been used. The agreement between several estimations on the same lactate solutions has been very nearly as good as in experiments where Schenk's method has been employed. (Within 0.02 cc. *N*/200 iodine on an average, as compared with an average variation of 0.01 cc. with Schenk's method.) Such improvement as there has been with the adoption of Schenk's method is thought to be due solely to increased skill in performing the estimations.

A. *Trichloroacetic acid method.* The procedure adopted was essentially the same as that of other workers [e.g. Kinnersley and Peters, 1930; Fisher, 1931; Ashford and Holmes, 1931]. The contents of each Barcroft bottle were treated with 0.5 cc. 20 % trichloroacetic acid immediately after the last reading of the oxygen uptake. The bottles were left in cold store overnight. The contents of the two control bottles were decanted into the same centrifuge-tube, and the bottles were washed out with three lots of 3 cc. of water. After centrifuging, the clear solution was poured off through a small filter-paper into a 200 cc. flask. The tissue in the tube was ground up with a glass rod in 0.5 cc. 20 % trichloroacetic acid and left to stand with frequent stirring for half an hour. 6 cc. of water were added with stirring, the whole was centrifuged, and the washings were poured off through the filter into the flask. The whole operation was repeated. The filter was then washed through with three or four lots of water. The bottles to which vitamin B₁ concentrate had been added were treated in exactly the same manner. To each flask was added 1 cc. 10 % copper sulphate solution and 1 cc. 20 % lime suspension (sufficient to make the solution alkaline). Both were made up to the mark, and left for an hour before filtering through small creased filter-papers. The first few cc. of the filtrates were rejected. Two solutions were thus obtained containing the lactate left by equal amounts of cerebrum, which had been placed in equal quantities of the same lactate solution, and treated with and without vitamin B₁ concentrate respectively.

B. *Schenk's method.* After the last reading 5 cc. 3 % HgCl₂ and 4 cc. 2 % HCl were added to each bottle. After standing overnight the bottles were washed out and their contents centrifuged as in the trichloroacetic acid experiments. The centrifugates were poured off into boiling-tubes, and the tissue left behind was twice ground up with glass rods and washed with 6 cc. of water. After centrifuging the washings were added to the boiling-tubes. To each tube about 0.5 cc. 20 % NaOH was added, thus bringing the p_H of the solutions to about 2. After treatment with H₂S for an hour and filtering into a vacuum flask, the filter was carefully washed, and air was drawn through the filtrate for several hours. The solution was then washed out into a 200 cc. flask, 3 drops of phenol red solution were added, and the solution was roughly neutralised with 20 % NaOH (about 0.8 cc.). Copper-lime treatment was carried out as before.

Percentage recovery of methods. Two previously estimated lactate solutions were treated by the above procedures. The Schenk method gave a recovery of 100 % in both cases. The trichloroacetic acid method gave yields of 94 and 95 %. The loss may arise in the initial filtration after removal of the precipitated proteins. No correction has been applied to the results for this loss, as it is not sufficient to affect them.

Lactate determinations. The method of Friedemann, Cotonio and Shaffer [1927], as modified by Friedemann and Kendall [1929], was employed. The apparatus was of usual type, with the exception of the absorption tower. This was made according to a design devised by Mr R. B. Fisher. It consisted of a narrow-bored tube containing a single tier of large glass beads retained by a constriction at the bottom. The tower was designed to take only 2 cc. of bisulphite solution, instead of the usual 10 cc. The solution employed was correspondingly more concentrated (4 %). By this method it was possible to wash out the bisulphite completely with only a small volume of water, and so to reduce the bulk of solution to be titrated at the finish. $N/200$ KMnO₄ was used for the oxidation, Na₂HPO₄ to liberate the bound bisulphite and $N/200$ iodine for the final titration. The iodine solution was made up fresh every day from a stock $N/10$ solution, which was checked at intervals against standard

thiosulphate. A battery of three apparatus was constantly employed, each estimation being performed in triplicate on aliquot samples, containing 0.2 to 0.5 mg. of lactic acid. When two solutions were compared (control and vitamin-treated), the estimation of one was performed directly after that of the other, and as nearly as possible under the same conditions. The blank titration of each apparatus was frequently checked, and the percentage yield of the estimation was determined at intervals on a standard zinc lactate solution. The method gave constant yields of $92 \pm 1\%$, although the conditions of Friedemann and Kendall for a maximum yield have been observed throughout. A correction has been applied to all experimental results to bring these values up to 100 %.

RESULTS.

1. Control and vitamin-treated avitaminous cerebrum in the presence of added lactate: oxygen uptake and lactate removal compared.

Table I.

Exp. No.	I Wet weight of tissue mg.		II Oxygen uptake in 3 hours cc.		III Lactate recovered after 3 hours mg. of lactic acid	
	V. C. Vitamin- Control treated		C. Vitamin- Control treated		C. Vitamin- Control treated	
	C.	V.	C.	V.	C.	V.
<i>Trichloroacetic acid experiments:</i>						
1	406	391	1.12	1.34	3.39	3.48
3	362	344	1.06	1.27	2.46	2.46
5	389	410	1.27	1.58	2.08	2.10
6	316	318	1.06	1.23	2.78	2.87
<i>Schenk experiments:</i>						
7	226	226	0.46	0.67	1.71	1.81
9	408	397	0.95	1.14	2.97	2.97
12	381	393	1.07	1.38	2.80	2.52
14	387	390	0.97	1.23	3.39	3.19

Table II.

Exp. No.	I Observed lactate difference mg. lactic acid C.-V. in Col. III, Table I		II Observed extra oxygen uptake cc. V.-C. in Col. II, Table I	III Expected lactate difference, "Lactic acid equivalent" of extra oxygen mg.	
	x			y	
1	-0.09	± 0.10	0.22	0.30	± 0.05
3	0	0.08	0.21	0.28	0.05
5	-0.02	0.10	0.31	0.41	0.07
6	-0.09	0.10	0.17	0.23	0.06
7	-0.10	0.05	0.21	0.28	0.03
9	0	0.02	0.19	0.25	0.06
12	+0.28	0.02	0.31	0.41	0.06
14	+0.20	0.04	0.26	0.35	0.07
Average	+0.02	± 0.06		+0.31	± 0.06

Cols. x and y represent the estimated maximum experimental errors in the figures in Cols. I and II respectively (see text).

The experimental results are given in Table I.

In Table II these results are compared. Col. I (of Table II) gives the observed differences in lactate left by the control and vitamin-treated halves of the mince. The differences in oxygen uptake are given in Col. II; these figures represent the extra oxygen uptake due to the addition of vitamin B₁ concentrate. Previous work has suggested that this extra uptake is concerned with the oxidative removal of lactate. The least amount of lactate that this extra oxygen could remove is the amount that it would completely oxidise, and this is given in Col. III. (1 cc. of oxygen would completely oxidise 1.34 mg. of lactic acid.)

The maximum probable experimental error in these results is given in Cols. *x* and *y*. This has been calculated as follows.

Errors. The error in weighing the tissue is negligible. An air-damped Sartorius balance was used throughout which is sensitive to 0.1 mg.

The lactate recovered from each sample of minced tissue is calculated from at least three separate lactate estimations in each case. No single estimation in the experiments quoted differed from the mean by more than 0.03 cc. N/200 iodine. The maximum errors in Col. *x* are obtained by taking the outside figures of each group of three estimations.

Each reading of the Barcroft apparatus is taken as being correct to within one scale division (about 3 mm.³). The final reading in all cases would then be correct to within $\pm 1\%$. The calibration constant of each apparatus has a probable error of $\pm 1\%$. The maximum error in the calculated oxygen uptakes is therefore $\pm 2\%$, and in the difference between two such estimations $\pm 4\%$. The "extra oxygen uptake" was 25% of the control uptake on the average, and so the average maximum error in the "lactic acid equivalent" of the extra uptakes is $\pm 16\%$. Calculated separately, the estimated maximum errors in these figures vary from ± 9 to $\pm 25\%$, and are given in Col. *y*. It must be emphasised that these are maximum errors. It is not likely that the actual errors have ever approached these figures.

Reference to Col. I of Table I shows that in some experiments there is an appreciable difference in the amount of tissue between the control and vitamin-treated portions of the mince. The difference is never more than 5%. Nevertheless this means that the observed extra oxygen uptake and difference in lactate removed is not an exact measure of the influence of the added vitamin B₁ concentrate.

A 5% excess of tissue in the control sample, for instance, will be sufficient to increase appreciably the oxygen uptake and the lactate removed above the figures that would be given by an amount of tissue exactly equal to the vitamin-treated sample. It will also increase the amount of lactate at the beginning of the experiment by virtue of the lactate preformed in the excess tissue. The error introduced by this difference in tissue may be accurately estimated by the following considerations. The oxygen uptake of the excess tissue will be proportional to its mass, and can be calculated from the uptake of the control sample.

Table II.

Figures corrected as described in the text.

Exp.	Observed lactic acid difference mg.	Expected lactic acid difference mg.
1	-0.05	+0.35
3	+0.04	+0.35
5	-0.07	+0.30
6	-0.09	+0.22
7	-0.10	+0.28
9	+0.02	+0.29
12	+0.26	+0.37
14	+0.19	+0.34
Average	+0.03	+0.31

The lactate removed by the excess tissue can be calculated from the results given in the next section. 1 g. of tissue has been found to remove about 4 mg. of lactic acid in 3 hours under these conditions. The preformed lactate in the excess tissue will increase the lactate at the start of the experiment by about 2 mg. per g. When the experimental results are corrected by these values, Table II then reads as above (p. 1315). These figures are subject to the same possible experimental errors as are the uncorrected figures.

It will be seen that the general nature of the results cannot be affected by any small differences that have occurred between the amounts of tissue in the control and vitamin-treated halves of the mince.

Reference to Col. I, Table II, shows that these experiments have failed to demonstrate any marked disappearance of lactate corresponding to the extra oxygen uptake induced by the addition of vitamin B₁ concentrate to minced avitaminous cerebrum. In eight experiments, only two have shown any greater removal of lactate by the vitamin-treated tissue as compared with the control, and in these the increase in lactate removed is less than the least amount that could be expected if the extra oxygen uptake induced by the vitamin B₁ concentrate were due to the oxidative removal of lactate. The differences in lactate removal between the control and vitamin-treated samples are of the order that might be expected from a slightly uneven distribution of enzymes in the minced tissue.

Four other experiments have been performed, which have not been included in the results given. They have been rejected on various grounds, chiefly because they were found to be susceptible to a rather wide margin of possible experimental error. In so far as any conclusions may be drawn from them they are in agreement with the foregoing results, and offer no evidence of any contrary behaviour.

There is one possible source of fallacy in the conclusions from these results which requires discussion. The production, or failure of oxidation, of some substance estimating as lactate in the vitamin B₁-deficient tissue, in the presence of added vitamin B₁ concentrate, might be sufficient to mask an actual increased oxidation of lactate. The possibility of lactate oxidation in the brain sparing other oxidations has received consideration from other workers. The method of Friedemann and Kendall reduces the number of substances which are estimated as lactate to very few. If the results were due to such a substance it would be necessary that enough should accumulate in 1 g. of tissue to be estimated as about 0.9 mg. of lactic acid. From the figures given by Friedemann, Cotonio and Shaffer this would require 4.5 mg. of cystine or 9 mg. of malic or glyceric acid. Most other substances would need to accumulate in much greater quantities. It is thought improbable that an increased lactate oxidation of the order that the added concentrate might induce could spare the removal of other substrates to this extent.

This investigation has been somewhat handicapped by the small size of the pigeon's brain. From the point of view of the estimations it would have been an advantage to perform large-scale experiments using several g. of minced cerebrum from a number of birds. Such a method has not been adopted for several reasons. In the first place it seemed possible that a mixture of tissue from several birds might introduce other variable factors, secondly it was desired to keep this research in line with previous work by using the same technique, and lastly it was impossible to rely on obtaining several birds in head-retraction at the same time. The experiments have therefore been performed on single cerebrums. The expected difference in lactate removal that has been investigated is admittedly small, and for this reason considerable care has been taken to define the accuracy of the results. It has been shown that the probable maximum errors are insufficient to affect the general nature of the results.

2. *The removal of lactate by avitaminous cerebrum.*

As a subsidiary problem the removal of lactate by the minced avitaminous cerebrum has also been studied in the absence of added vitamin B₁ concentrate.

Table III. *Minced cerebrum of polyneuritic pigeon incubated for 3 hours in the presence of added lactate.*

All lactate figures given in terms of mg. of lactic acid. Lactate preformed in the tissue taken as 2 mg. per g. of tissue. Schenk's method used in the extraction of lactate.

Exp. No.	I Wet weight of tissue g.	II Esti- mated lactate at start	III Lactate recovered after 3 hours	IV Estimated lactate removed in 3 hours	V Oxygen uptake in 3 hours cc.	VI "Lactic acid equivalent" of oxygen uptake
7	0.23	2.42	1.71	0.71	0.46	0.60
8	0.35	4.61	3.06	1.55	0.95	1.27
9	0.41	4.71	2.97	1.74	0.95	1.27
10	0.34	4.57	2.90	1.67	0.99	1.32
11	0.38	4.48	2.92	1.56	1.08	1.44
12	0.38	4.50	2.80	1.70	1.07	1.43
13	0.30	4.90	3.68	1.22	0.72	0.96
14	0.39	5.08	3.39	1.69	0.98	1.31

The experimental results are given in Table III. The table has been compiled from the figures of control samples of cerebrum in experiments set up primarily for the investigation of the problem discussed in the previous section. Exactly the same methods have been employed as have been previously described. Schenk's method of protein precipitation was used. The lactate solution added to the tissue was previously accurately estimated. In calculating the amount of lactate at the start of the experiment allowance must be made for the lactate preformed in the tissue itself. Unfortunately there was never sufficient tissue left over after filling the Barcroft bottles to provide enough for an accurate estimation of the preformed lactate.

Kinnersley and Peters [1929; 1930] have shown that the amount of lactate in avitaminous pigeon's brain 1 minute after death is about 1.5 mg. per g. of tissue on the average. Thereafter little further increased formation of lactate seems to occur in the tissue, even after incubation for 1 hour in Ringer at 38°. The highest figure found by them for avitaminous brain was 2.3 mg. per g.

As a safe figure the total amount of lactate provided by the tissue itself can be taken as 2 mg. per g. (with a possible variation of 0.5 mg.). In Col. II of Table III an allowance for this amount of preformed lactate has been made in the estimated total lactate at the start of the experiment.

Col. III gives the lactate recovered after incubating the tissue for 3 hours in oxygen. This is calculated from the mean of at least three lactate estimations in each case.

Col. IV gives the estimated lactate removed (Cols. II-III).

For the purposes of comparison the total oxygen uptake is given in Col. V, and in Col. VI the "lactic acid equivalent" of the oxygen uptake (that is, the amount of lactic acid that it would completely oxidise).

It will be seen that the total oxygen uptake would be only sufficient completely to oxidise from 75 to 90 % of the lactate apparently disappearing. If the preformed lactate in the tissue is taken as 1.5 mg. per g. instead of 2 mg., the oxygen taken up could completely oxidise 85 to 100 % of the disappearing lactate.

An important factor in these experiments is the efficiency of the extraction of the lactate from the tissue. If the Schenk precipitate retained a significant amount of lactate, there would be an exaggeration of the apparent lactate removed.

Previously estimated lactate solutions have therefore been added to minced brain under the conditions of an actual experiment and the mixture treated for lactate estimations as described previously. The following were the results obtained.

	Tissue added g.	Lactate added mg. of lactic acid	Lactate recovered
Normal cerebrum	0.11	3.60	3.71
" "	0.10	1.42	1.48
Avitaminous cerebrum	0.10	1.83	2.02

The lactate recovered is greater than the lactate added by an amount of the same order as the probable preformed lactate in the tissue.

The experimental results of this section cannot therefore be attributed to experimental loss of lactate during the process of extraction.

In view of the absence of direct determinations of the lactate preformed in the tissue, too much stress should not be laid on the figures given in Table III. Nevertheless these experiments show beyond doubt that minced vitamin B₁-deficient cerebrum can remove a considerable quantity of lactate (1.40 mg./g. of tissue/hr. on the average). This would require for its complete oxidation at least the whole oxygen taken up by the tissue.

This presents the same problem as the work of Ashford and Holmes [1931] on rabbit's brain, and, in the same way, suggests a removal of lactate by some path other than direct oxidation.

The large removal of lactate seems to make it still more probable that there is no defect in the removal of lactate by the minced vitamin B₁-deficient brain¹.

DISCUSSION.

The results of this research indicate that the nature of the lesion in vitamin B₁-deficient brain is less simple than previous evidence suggested. The lesion results in a lowered oxygen uptake of the minced brain in the presence of lactate. The addition of vitamin B₁ concentrate to the minced brain largely restores the lowered uptake and yet causes no corresponding increase in the removal of lactate. The vitamin B₁ concentrate must be concerned with the oxidation of some substance other than lactate itself, although its action is in

¹ The investigation of the removal of lactate by the normal brain is not part of the object of this research. Two preliminary experiments however suggest that it is of the same order as in the vitamin B₁-deficient brain. The results of these experiments are given below.

	Normal minced cerebrum				Lactic acid equivalent of oxygen uptakes (mg.)	
	Duration of incubation in oxygen	Wet weight of tissue mg.	d-Lactic acid added mg.	Lactic acid removed mg.	Total uptake	Extra uptake due to lactate addition
Bird A:						
Sample I	90 mins.	120	3.60	0.35	0.47	0.18
" II	90 "	130	3.60	0.28	0.48	0.18
Bird B:						
Sample I	120 mins.	100	1.42	0.42	0.45	0.22
" II	120 "	100	1.42	0.32	0.40	0.19

some way dependent on the presence of lactate¹. The evidence of previous work can leave little doubt that vitamin B₁ concentrate specifically restores the oxidation that is defective in the minced vitamin B₁-deficient brain². If this evidence is accepted, it must be concluded that this defect in oxidation does not interfere with the removal of lactate itself in the minced brain. On the other hand the accumulation of lactate in the living brain in vitamin B₁ deficiency [Kinnersley and Peters, 1929; 1930] suggests that the removal of lactate is defective *in vivo*. It has been suggested previously that lactate may be removed in the brain by some path other than direct oxidation. The lesion in vitamin B₁-deficient brain may affect an oxidation at some essential stage in the metabolism of lactate subsequent to its initial removal. This might result in the accumulation of lactate in the living brain; while under the artificial conditions of minced brain *in vitro*, it might have no influence on the initial removal of lactate, but result instead in the continuous formation and accumulation of the product of lactate whose oxidation is impaired by the lesion. The evidence can be interpreted in other ways, but it is thought that this forms the most simple explanation at the present time.

It is concluded from this research that the lesion in vitamin B₁-deficient brain affects an oxidase system that is associated with lactate, but is not concerned with the removal of lactate itself in isolated brain tissue.

SUMMARY.

1. Previous work has shown that the oxygen uptake of minced pigeon's brain in the presence of lactate in vitamin B₁ deficiency is lower than the normal. The addition *in vitro* of vitamin B₁ concentrate, in small amounts, largely restores the defect.

2. It is here shown that the addition *in vitro* of small amounts of vitamin B₁ concentrate, while increasing the oxygen uptake of the avitaminous cerebrum in the presence of added lactate causes no significant increase in the amount of lactate removed.

3. The minced cerebrum of the vitamin B₁-deficient pigeon readily removes lactate *in vitro*. The amount removed in 3 hours is sufficient to require at least the total oxygen taken up by the tissue for its complete oxidation.

I wish to express my most sincere thanks to Prof. Peters for his constant encouragement and advice during the course of this research. I also wish to thank Mr R. B. Fisher for his advice on the method of performing the lactic acid estimations and for his assistance in the preparation of the specimen of *D*-lactate used. I am also indebted to Mr Kinnersley for supplying the vitamin concentrate.

¹ In previous experiments [Gavrilescu *et al.*, 1932] it was shown that the addition of vitamin B₁ concentrate *in vitro* increased the oxygen uptake of vitamin B₁-deficient cerebrum by 145 mm.³/g./hr. on the average in the absence of added substrates, and 340 mm.³/g./hr. in the presence of added lactate. The small increase in the absence of added substrates was attributed to the presence of lactate preformed in the tissue itself.

² The addition of vitamin B₁ concentrate to the avitaminous brain in the presence of succinate, and to the normal brain with lactate, causes no significant increase in the oxygen uptake [Gavrilescu *et al.*, 1932]. Furthermore in birds recovering from polyneuritis the effect of the concentrate in the presence of lactate decreases as the oxidative behaviour of the brain with lactate improves [Meiklejohn *et al.*, 1932].

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CLXXIX. A COMPARATIVE INVESTIGATION OF URINE- AND SERUM-PROTEINS IN NEPHRITIS.

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(Received June 30th, 1933.)

WHILST various attempts have been made to solve the problem as to whether the passage of protein through the kidney involves structural alteration of the molecule, no complete investigation on the subject has been carried out.

Hynd [1925] found close agreement in specific rotation between the serum- and urine-albumin in the albuminuria of pregnancy except for certain cases of eclampsia in which the urinary albumin was apparently identical with cow's lactalbumin. His results, however, are not reliable since his proteins were not entirely lipid-free, and the solutions which he used for the optical rotation measurements were so dilute that in some cases his experimental error was probably nearly 30 %.

Hewitt [1927, 1; 1929] observed that albumins from the serum and urine of patients with chronic nephritis and albuminuria were identical in optical rotatory power and concluded that serum-albumin probably leaks through the kidney unchanged. He could not confirm the results of Hynd [1925]. Hewitt's method of separation of the albumins from serum and urine appears to be satisfactory, but his investigation of these proteins was confined to observations of their specific rotatory powers. Also, this worker makes no attempt to isolate his albumins in the solid form, but relies upon determinations of nitrogen in their solutions for the calculation of the protein concentration, apparently assuming that the proteins are sufficiently pure for the nitrogen percentage in them to be taken as equal to the theoretical value.

A study of the physical properties of proteins as a means of their identification has been carried out by various workers.

Hardy and Gardiner [1910] and Young [1922] have shown that the optical rotation of proteins differs with variations in the p_H of the solution, and Woodman [1921] has demonstrated that the optical rotation of a protein in alkaline solution falls for about 2 weeks. If the rotation is plotted against time, a definite curve is obtained. This worker suggests that this racemisation curve is a most satisfactory means of identifying a protein.

Cavett and Gibson [1931] have shown that the racemisation curves given by albumins and pseudoglobulins from nephritic urines are similar to those of the corresponding serum-proteins. These workers, however, appear to have assumed that a single fractional precipitation is sufficient completely to separate the albumin and the globulin in the mixed protein. Experience has shown that at least three or four fractional precipitations with ammonium sulphate are necessary if the proteins are to be obtained in a purified form, and it is of interest to note that all proteins relied upon by Woodman for his work on racemisation were fractionally precipitated seven times, while Hewitt [1927, 1, 2; 1929], who investigated the optical rotation of urinary albumin, precipitated his protein three times with ammonium sulphate.

Govaerts [1924; 1927; 1929] and Verney [1926] have investigated the osmotic pressures of the serum-proteins from nephritic patients. Both these workers, however, appear to have confined their attention to a study of the osmotic pressure of whole serum, and no evidence of an attempt to determine the osmotic pressure of solutions of the separated serum-proteins could be found in the literature.

Schretter's [1926] survey of previous work on the specific refractions of proteins in normal and pathological sera shows that the results obtained by various workers differ widely one from another, probably owing to the impure proteins employed by the different workers. Adair and Robinson [1930] have carried out a very careful study of the specific refraction increments of recrystallised albumin and globulin from horse-serum, and they have shown that the value is a constant for any given protein.

It was thought that it would form a most interesting study to investigate the identity of the urine- and serum-proteins of nephritic patients by means of the Van Slyke [1911] nitrogen distribution method and further to examine these proteins by a study of their racemisation curves, osmotic pressures and specific refractions. It was hoped that by combining the results obtained by chemical and physical means to obtain definite information as to whether the protein is excreted as a foreign body or whether it leaks through the kidney unchanged.

Source of material.

A normal healthy man was selected as a source of material for the normal plasma-proteins, whilst the blood and urine in cases of proteinuria were obtained from in-patients of the Middlesex Hospital.

S. B. This patient was a man, aged 57, who had been under observation for some 3 years. He was a typical case of nephrosis with marked oedema, a high blood-cholesterol and massive proteinuria. During the period that the specimens were collected there was no evidence of interstitial changes in the kidney. The patient subsequently developed these, and at autopsy the kidney showed severe parenchymatous degeneration with superadded interstitial changes.

The other four cases, *H. P.*, *H. F.*, *D. J.* and *E. F.*, were suffering from what would be described as chronic parenchymatous nephritis associated with long-standing proteinuria. The patients had all given a history of acute or sub-acute nephritis, and were clinically and biochemically distinct from the case of *S. B.*

Preparation of proteins.

Urine-proteins. The method employed for the precipitation and purification of the urine proteins was similar to that used by Hewitt [1929]. The fresh urine was filtered and 2.3 times its volume of saturated ammonium sulphate solution was added. The p_H was adjusted to 4.7 by the addition of acetic acid, and the precipitation was allowed to continue overnight. The precipitate was then separated by means of the Sharples centrifuge, ground up with a small quantity of water and added gradually and with constant shaking to 10 times its volume of a mixture of absolute alcohol (7 volumes) and ether (3 volumes) cooled to a temperature of -5° . The mixture was allowed to stand at a low temperature overnight and the precipitated proteins filtered off on a Büchner funnel, washed thoroughly, first with alcohol and ether mixture and finally with ether alone. The precipitate was then transferred to a Soxhlet apparatus and extracted with dry ether for 24 hours, any alcohol present in the ether being removed by the presence in the flask of a small piece of sodium. Both the ether and the sodium were renewed at the end of the first 2 hours. When the extraction was complete the proteins were spread in the air to dry.

Serum-proteins. The serum, prepared by the usual laboratory method, was poured slowly into 10 times its volume of a mixture of alcohol (7 parts) and ether (3 parts) cooled to -5° as recommended by Hewitt [1927, 1, 2]. The precipitation was allowed to proceed to completion overnight, and the precipitated proteins were treated in a manner similar to that described for the urine-proteins.

Separation of albumin and globulin.

Urine-proteins. The mixed proteins were ground in a mortar with a small quantity of distilled water until they had become thoroughly moistened, the quantity of water being gradually increased to a suitable volume, and the solution was then neutralised by the addition of a few drops of ammonia. The clear solution thus obtained was filtered from the small amount of insoluble material which remained and treated with half its volume of saturated ammonium sulphate solution. After the precipitation had continued overnight the globulin was filtered off on a fluted paper and the filtrate half-saturated with ammonium sulphate. In most cases there was no further precipitate, but where a turbidity was produced the solution was filtered and the precipitate discarded. The precipitation of globulin at $\frac{1}{2}$ saturation with ammonium sulphate, instead of at the more usual $\frac{1}{3}$ saturation, lessens the possibility of contamination of the precipitate with albumin, since the dry mixed protein contains a considerable percentage of ammonium sulphate which increases the concentration of this salt beyond the calculated value.

Sufficient ammonium sulphate solution was next added to the filtrate to render the final concentration of the salt 70 %, and the p_H was adjusted to 4.7 by the addition of acetic acid. Next morning the precipitated albumin was filtered off on a fluted paper. The globulin and albumin were redissolved in water and reprecipitated 3 times at concentrations of $\frac{1}{3}$ and 70 % saturation with ammonium sulphate.

After the final filtration the precipitates were taken up with water and the solutions dialysed through cellophane bags against running water for 3 days, then against distilled water which was changed daily until the protein solution was free from ammonia. The dialysed solution was filtered and evaporated down to small bulk *in vacuo* at 40°, the solution being allowed to drop into the distilling flask, from a funnel drawn out to a capillary, as fast as the water was removed by distillation. The concentrated solution of pure protein was added drop by drop to a mixture of alcohol (7 parts) and ether (3 parts) cooled to -5° as before, and, after standing for some hours, the proteins were filtered off on a Büchner funnel, dried *in vacuo* over sulphuric acid and weighed.

Serum-proteins. The precipitated serum-proteins were separated by a method similar to that employed for the urine-proteins, but all precipitations were carried out in a 100 cc. centrifuge-tube, and the precipitates were centrifuged instead of being removed by filtration. Globulin was precipitated 4 times at $\frac{1}{3}$ saturation with ammonium sulphate. After each precipitation of the globulin, the concentration of ammonium sulphate was increased to 50 %. In some cases a very small precipitate was formed but never in sufficient quantity to warrant further investigation. It was therefore removed by filtration, the ammonium sulphate concentration was increased to 70 % and the albumin precipitate centrifuged off, taken up with water and reprecipitated 3 times. The solutions of the separated proteins were dialysed as before, then filtered, and the water was removed by leaving the solutions in a vacuum desiccator over sulphuric acid for about a week.

It will be observed that the above method of isolation of the proteins from serum or from urine makes no attempt to separate the two globulins, euglobulin and pseudoglobulin, by fractional precipitation. According to various workers [see Hartley, 1914; Woodman, 1921; Cavett and Gibson, 1931] euglobulin is precipitated at $\frac{1}{3}$ saturation with ammonium sulphate, and pseudoglobulin at $\frac{1}{2}$ saturation with this salt. It has already been pointed out, however, that it is difficult to ascertain exactly the ammonium sulphate concentration of the solution during the process of fractional precipitation, and it was thought that it would be more satisfactory to carry out analyses on the total globulin rather than to attempt a separation of the two globulins which would certainly be most unreliable. Hartley [1914] has shown that analyses of euglobulin and pseudoglobulin failed to reveal any striking differences in the chemical composition of these two substances.

Analysis of urine- and serum-proteins by the Van Slyke nitrogen distribution method.

The small amounts of serum-albumin and globulin available for analysis necessitated the use of a micro-method for the determination of the Van Slyke [1911] nitrogen distribution. The procedure adopted was that of Narayana and Sreenivasaya [1928] with the modifications that the ammonia-N was determined by the method of Plimmer and Rosedale [1925] and the arginine-N by a micro-adaptation of the method of Koehler [1920].

Table I. *Comparative analyses of urine-albumin by the micro- and macro-methods of determination of the nitrogen distribution.*

(Results expressed as percentage of total N.)

	Duplicate analyses by the micro-method		Analyses by Plimmer and Rosedale's modification of Van Slyke's method
	1	2	
Ammonia-N	6.63	6.28	6.40
Humin-N	1.12	1.25	1.49
Arginine-N	7.99	7.82	7.54
Histidine-N	7.01	6.53	5.09
Cystine + lysine-N	14.44	14.77	20.50
Amino-N of filtrate	60.34	61.16	58.29
Non-amino-N of filtrate	2.02	1.88	1.53
Total N recovered	99.55	99.69	100.84

The results of these determinations are shown in Table I and it will be observed that the agreement between the results for the duplicate analyses by the micro-method is fairly close. Apart from the histidine, which is a calculated percentage and which is therefore most liable to error, the variation is always less than 0.5 % of the total nitrogen. Certain differences between the results of the analyses by the micro- and macro-methods are, however, apparent. The micro-method gives results for the cystine- and lysine-nitrogen which are about 6 % lower than those obtained by the macro-method, while the value for the non-basic nitrogen is definitely higher. This difference is explained by the fact that the precipitation of the bases in the micro-method is carried out in a more dilute solution than when the experiment is performed on a macro-scale. Hence a larger correction for the solubility of the bases must be applied in the case of the small scale experiment. Since no figures are available for this correction, and since it did not fall within the scope of this work to determine them, it was decided that in order to obtain comparative results for the analyses of the urine- and serum-proteins it would be necessary to carry out all the analyses by the micro-method.

The results of these analyses are shown in Tables II and III, being expressed as percentages of the total nitrogen in the protein.

It will be observed that there are no very striking differences between the values obtained for urine-protein and those of the corresponding serum-protein in patients suffering from nephritis or nephrosis. From this it may be concluded that there is no chemical alteration in the structure of the protein during its passage through the kidney.

Table II. *Analyses of urine- and serum-albumins by the Van Slyke nitrogen distribution method.*

(Results expressed as percentage of total N.)

	S. B.		H. P.		H. F.		D. J.		E. F.	Normal serum-albumin
	Urine-albumin	Serum-albumin	Urine-albumin	Serum-albumin	Urine-albumin	Serum-albumin	Urine-albumin	Serum-albumin	Urine-albumin	
Ammonia-N	6.63	6.25	7.66	7.08	6.97	7.13	7.40	7.37	7.38	7.31
Humin-N	1.12	1.48	0.70	1.50	0.34	0.73	0.40	0.83	0.66	0.47
Arginine-N	7.99	28.93	7.80	8.01	8.33	7.83	8.64	8.57	8.00	8.22
Histidine-N	7.01		7.52	7.68	6.32	6.56	6.34	5.76	7.66	6.83
Cystine + lysine-N	14.44		11.75	11.25	13.44	12.18	11.93	11.93	11.76	12.38
Amino-N of filtrate	60.34	60.86	61.08	61.88	60.97	61.57	61.33	61.93	59.89	60.60
Non-amino-N of filtrate	2.02	2.97	3.16	2.43	2.86	3.37	2.94	3.04	3.54	2.55
Total N recovered	99.55	100.49	99.67	99.83	99.23	99.37	98.98	99.43	98.89	99.36

Table III. *Analyses of urine- and serum-globulins by the Van Slyke nitrogen distribution method.*

(Results expressed as percentage of total N.)

	S. B.		H. P.		H. F.		D. J.		E. F.	Normal
	Urine-globulin	Serum-globulin	Urine-globulin	Serum-globulin	Urine-globulin	Serum-globulin	Urine-globulin	Serum-globulin	Urine-globulin	serum-globulin
Ammonia-N	11.46	11.89	10.42	8.65	9.80	9.27	9.02	9.04	9.79	9.25
Humin-N	0.96	1.52	1.70	0.41	1.10	0.56	0.82	0.74	1.62	0.53
Arginine-N	9.70	10.07	10.22	10.01	10.20	10.49	11.46	11.57	10.98	10.67
Histidine-N	4.72	5.18	5.21	4.52	5.24	4.23	2.54	2.80	2.96	4.21
Cystine + lysine-N	7.16	7.08	7.78	9.63	8.31	7.46	9.86	10.33	9.28	9.78
Amino-N of filtrate	63.37	61.46	58.70	60.88	60.82	63.82	63.52	61.93	58.22	61.32
Non-amino-N of filtrate	3.08	3.16	5.43	5.84	3.58	3.71	2.76	2.76	6.70	3.57
Total N recovered	100.45	100.36	99.46	99.94	99.05	99.54	99.98	99.17	99.55	99.33

A comparison of the analyses of the proteins from different patients shows some very interesting results. The albumins and globulins in the urine and serum of patients suffering from nephritis are apparently identical with those of normal human serum. In the case of nephrosis, however, certain slight differences are apparent. The corresponding urine- and serum-proteins are identical, but the analyses of these differ in certain respects from those of the proteins from normal serum or from the urine or serum of patients who are suffering from nephritis. The most marked difference is seen in the percentage of ammonia-N. This in the case of the urine- and serum-albumin from a patient suffering from nephrosis is about 1 % lower than the mean value obtained from normal serum-albumin and nephritic urine- and serum-albumin. The corresponding value for globulin in nephrosis is about 2 % higher than the normal value. Slight differences are also noted in the percentage of nitrogen in the basic and non-basic fractions. The basic nitrogen for albumin has a value 2 % higher in the case of nephrosis, while the basic nitrogen in the globulin is about 2 % lower than in the normal protein. These differences are small, and since only one case of nephrosis was studied, no definite statement can be made regarding this apparent slight alteration in the chemical structure of the urine- and serum-proteins in nephrosis.

The racemisation curves of urine- and serum-proteins.

The racemisation of the proteins was investigated by a method similar to that employed by Cavett and Gibson [1931].

About 0.4 g. of the urine-protein was shaken with 10 cc. of distilled water and left to stand overnight. 10 cc. of *N* NaOH were then added, and the mixture was well shaken and filtered through a pad of asbestos in a small Gooch crucible. By these means about 20 cc. of a clear solution consisting of 2 % protein in *N*/2 NaOH were obtained.

Polarimeter readings were taken at intervals of a few hours during the first day, and subsequently only once every 24 hours, and the racemisation was allowed to proceed for 10 days. A Schmidt and Haensch polarimeter was employed, and a Zeiss electric sodium vapour lamp was used as a source of light ($\lambda = 5893 \text{ \AA.}$). This lamp, whose intensity is about 50–100 times as great as that of a Bunsen burner with rock salt, enabled the polarimeter readings to be taken with ease and accuracy, and without any undue strain on the eyes.

After each reading, the 10 cc. of protein solution in the polarimeter-tube were added to the remainder of the same solution in a test-tube, which was then stoppered and incubated at 38° throughout the racemisation period. When

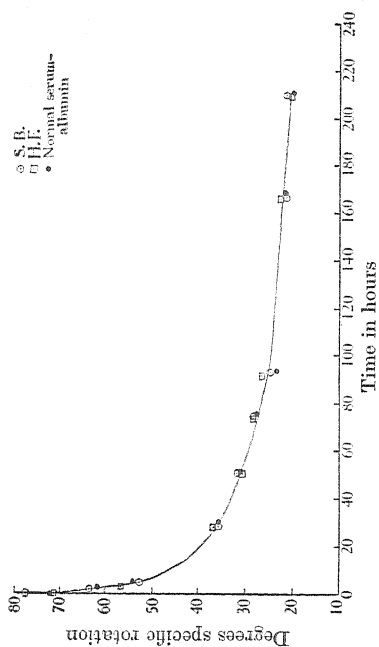


Fig. 2. Racemisation of serum-albumins in alkaline solution.

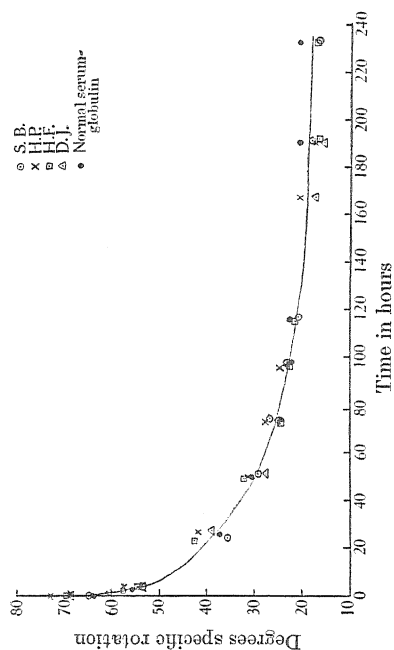


Fig. 4. Racemisation of serum-globulins in alkaline solution.

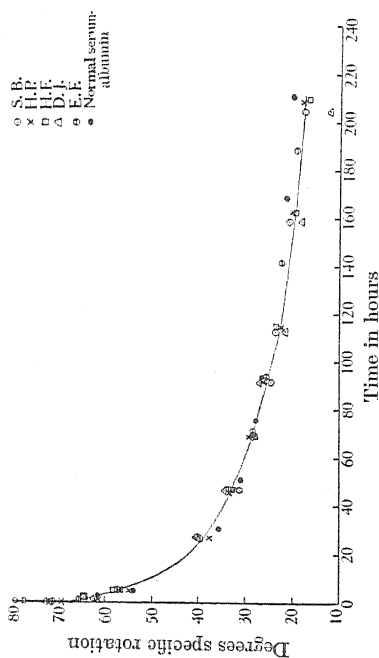


Fig. 1. Racemisation of urine-albumins in alkaline solution.

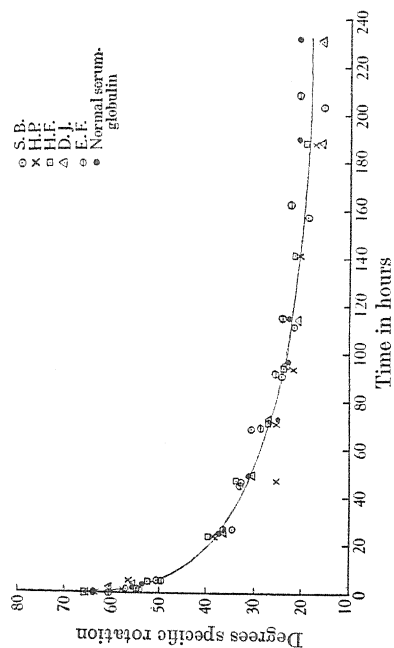


Fig. 3. Racemisation of urine-globulins in alkaline solution.

it was desired to take another reading, the solution was removed from the incubator, cooled in running water, and the clean dry polarimeter-tube was refilled. This procedure makes it possible to carry out a series of readings on solutions of several proteins simultaneously, even though only one polarimeter-tube is available. The 20 cc. of protein solution originally prepared were sufficient to fill the tube 10 or 11 times provided that care was taken during the filling process.

In the case of the serum-proteins, where only a small amount of material was available, a micro-polarimeter-tube was employed, 8 cm. in length and of 0.15 cc. capacity.

About 0.1 g. protein was dissolved in 5 cc. $N/2$ NaOH and the solution was filtered. The polarimeter-tube was freshly filled between each reading as before.

The specific rotatory power of the protein at each stage during the racemisation period was calculated from the mean value of 10 polarimeter readings. At the end of the experiment an aliquot portion of the protein solution was diluted to 5 times its volume and the total N determined on duplicate portions of 2 cc. by the micro-Kjeldahl method. The percentage of total N in the dry protein having been previously estimated, the concentration of protein in the solution could be calculated. From these results the specific rotatory powers could be determined, and these are shown in Figs. 1-4 where specific rotatory power is plotted against time of racemisation.

There is evidently no striking difference between the optical rotation of the urine-protein and that of the corresponding serum-protein in patients suffering from nephritis or nephrosis. These results confirm those which were obtained from the chemical investigation of the same proteins and indicate that there is no alteration in the structure of the protein during its passage through the kidney.

It will be observed from Fig. 1 that the points plotted for the racemisation of urine-albumin from five different patients suffering from nephritis or nephrosis all lie on the same curve as those for normal serum-albumin. Similar results are obtained for the serum-albumins (Fig. 2), the urine-globulins (Fig. 3) and the serum-globulins (Fig. 4). Hence the albumins and globulins in the urine and serum of patients suffering from nephritis are apparently identical with those of normal human serum, so far as their optical rotations are concerned. Certain slight differences in the chemical structure of the proteins in the case of nephrosis have already been noted. A similar difference was not apparent in the optical properties of the proteins. The albumins and globulins from the urine and serum of a patient suffering from nephrosis are apparently physically identical with those of normal human serum.

It is probable therefore that in nephrosis there is some alteration in the arrangement of the amino-acids in the serum- and urine-proteins, but this difference is not detectable by a measurement of their optical rotations.

Measurement of the osmotic pressures of urine- and serum-proteins.

The apparatus used for the measurement of the osmotic pressures of solutions of albumin and globulin from urine and serum was similar to that described by Verney [1926], except that one osmometer only was used, instead of the series employed by him. A water manometer was used to measure the osmotic pressure, and a disc of cellophane (substance 300) was employed as the semi-permeable membrane.

The precautions which Verney took to sterilise the parts of his apparatus

were found to be unnecessary, when, instead of serum, a solution of purified protein was used. The metal parts were coated with a thin layer of paraffin wax before the commencement of the experiment.

The albumin or globulin was dissolved in 0.9 % sodium chloride solution so that the protein concentration was approximately 6 %, and 0.9 % sodium chloride solution was used as the external liquid. Verney's procedure was followed exactly, and the osmosis was allowed to continue for 24 hours. After taking the manometer reading the protein solution was removed and the total N determined. Duplicate osmotic pressure determinations for each protein were made, and the results were expressed as osmotic pressure (cm. water) per g. protein per 100 cc. They are shown in Table IV.

Table IV. *Osmotic pressures of urine- and serum-proteins.*

(Calculated per g. protein per 100 cc.)

	O.P. of urine- albumin	Temp. ° C.	O.P. of serum- albumin	Temp. ° C.	O.P. of urine- globulin	Temp. ° C.	O.P. of serum- globulin	Temp. ° C.
S. B.	8.810	15	8.973	16	1.704	16	1.763	18
H. P.	9.400	17	—	—	1.796	16	1.754	17
H. F.	7.868	15	8.240	18	1.758	15	1.745	18
D. J.	8.596	16	8.058	17	1.719	16	1.678	17
E. F.	8.775	15	—	—	1.765	15	—	—
Normal	—	—	8.862	17	—	—	1.769	18

It will be observed from the results that the osmotic pressure per g. urine-albumin per 100 cc. is almost identical with the osmotic pressure of the corresponding serum-albumin, and similar results are obtained for the urine- and serum-globulins. The variation in the values for the osmotic pressures of the proteins from the different patients is very small. A mean value of 1.75 cm. water per g. protein per 100 cc. is obtained for the osmotic pressure of urine- and serum-globulins, while the corresponding value for the albumins is about 8.65 cm.

Govaerts [1927] obtained values for the osmotic pressures of serum-globulin and albumin of 1.95 and 7.54 cm. water respectively. His values were not obtained by direct measurement however but were calculated from a knowledge of the osmotic pressure, the protein-N content and the albumin/globulin ratio of the serum. Von Farkas [1927] obtained values of 2.51 and 6.80 cm. water per g. globulin and albumin per 100 cc. respectively.

The results shown in Table IV for the osmotic pressure per g. globulin per 100 cc. are a little lower than those obtained either by Govaerts or by von Farkas, while the corresponding values for albumin are slightly higher than those found by these other workers.

Govaerts [1927] states that the values obtained by him are merely empirical values, obtained as the result of observations, and must not be regarded as physico-chemical constants. Howe's [1924] method, which was employed by Govaerts for the determination of the albumin/globulin ratio, cannot be considered to be quantitatively accurate. A small error in the protein separation will make a considerable difference to the albumin/globulin ratio and consequently to the calculated osmotic pressure for each protein. The method employed in the present instance for the fractional precipitation of the urine- and serum-proteins and the use of solutions of the separated proteins for the osmotic pressure determinations eliminate all error due to an incomplete separation, and the osmotic pressure measurements are accurate to within 3 %. Govaerts considers his results to have an accuracy of 10 %.

The specific refractions of urine- and serum-proteins.

The specific refractions of the urine- and serum-proteins were determined by means of a Zeiss portable interferometer. The instrument was first calibrated by means of sodium light, but white light was used for all subsequent measurements. The proteins were ground up with 0.9 % sodium chloride solution, filtered through a pad of asbestos, and the clear solutions were used for the specific refraction determinations. Sodium chloride solution, of exactly the same strength as that used for dissolving the protein, was placed in the comparison chamber of the apparatus. After the interferometer readings had been taken, total N was determined on duplicate 1 cc. portions of the protein solution, and from the results the percentage of protein in the solution was calculated.

The specific refraction of each solution was calculated and hence the specific refraction of a 1 % solution of protein in 0.9 % sodium chloride solution could be determined.

Table V. *Specific refractions of urine- and serum-proteins.*

(Calculated for a 1 % solution.)

	Urine-albumin	Serum-albumin	Urine-globulin	Serum-globulin
S. B.	0.001965	—	0.002053	0.002132
H. P.	0.001982	—	0.001923	0.002067
H. F.	0.002000	0.001977	0.001928	0.001905
D. J.	0.001992	0.002071	0.002261	0.002198
E. F.	0.001914	—	0.002155	—
Normal	—	0.001968	—	0.002053

The results of these measurements are shown in Table V, and it will be observed that there are no striking differences between the specific refractions of the urine- and serum-albumins, or of the urine- and serum-globulins. The values for the albumins vary over a very small range, from 0.00191 to 0.00207. The variation in the values for the urine- and serum-globulins is somewhat greater, the figures ranging from 0.00191 to 0.00226. The values obtained for the urine- and serum-proteins of any one patient, however, are in close agreement.

Adair and Robinson [1930] obtained a mean value of 0.00181 for the specific refraction increment of recrystallised horse serum-albumin, while their corresponding value for globulin was 0.00186. Other workers [Starlinger and Hartl, 1925; Schretter, 1926] have investigated human serum-proteins and have obtained a mean value of 0.00200 for the specific refraction of albumin and 0.00219 for human serum-globulin.

Thus, the results obtained from the present experiments agree fairly closely with those obtained by other workers.

The variation in the specific refraction of the proteins studied is sufficiently small to warrant the conclusion that there is no alteration in the physical structure of the serum-proteins in nephritis or in nephrosis so far as can be determined by measurements of their specific refractions.

DISCUSSION.

The results of the investigations which have been made on the nitrogen distribution, racemisation, osmotic pressure and specific refraction of urine- and serum-proteins from nephritic patients indicate that the corresponding urine- and serum-proteins from any one patient are identical.

In ordinary nephritis and in prolonged proteinuria there appears to be no alteration in the structure of the proteins. This fact seems to decide once and for all that there is no defect in protein synthesis in these cases.

As judged by physical methods the proteins from patients suffering from nephritis and nephrosis appear to be the same. Chemically, however, they are distinct, though the differences noted are not very great. Since only one case of nephrosis was studied, no great emphasis can be laid on this slight difference in chemical structure.

Since the physical characteristics of the proteins are the same in all cases studied, it appears unlikely that these slight chemical differences in the case of nephrosis are of any clinical importance.

SUMMARY.

1. The albumins and globulins from the urine and serum of nephritic patients, and from normal human serum, have been separated by fractional precipitation with ammonium sulphate.

2. The separated albumins and globulins were analysed by a micro-modification of the Van Slyke nitrogen distribution method. Measurements of the optical rotation and racemisation, osmotic pressure and specific refraction of the proteins have also been made.

3. The results of these analyses indicate that the proteins isolated from the urine of patients suffering from nephritis or nephrosis are identical with the corresponding serum-proteins and there is no evidence of any alteration in the chemical or physical structure of the proteins during their passage through the kidney.

4. The proteins isolated from the urine and serum of patients suffering from nephritis appear to be identical in chemical and in physical structure with those of normal human serum.

5. The urine- and serum-proteins of a patient suffering from nephrosis exhibit certain slight differences in chemical structure from the normal serum-proteins. The racemisation curves, osmotic pressures and specific refractions of the urine- and serum-proteins of a patient suffering from nephrosis, however, appear to be identical with those of the proteins from normal human serum.

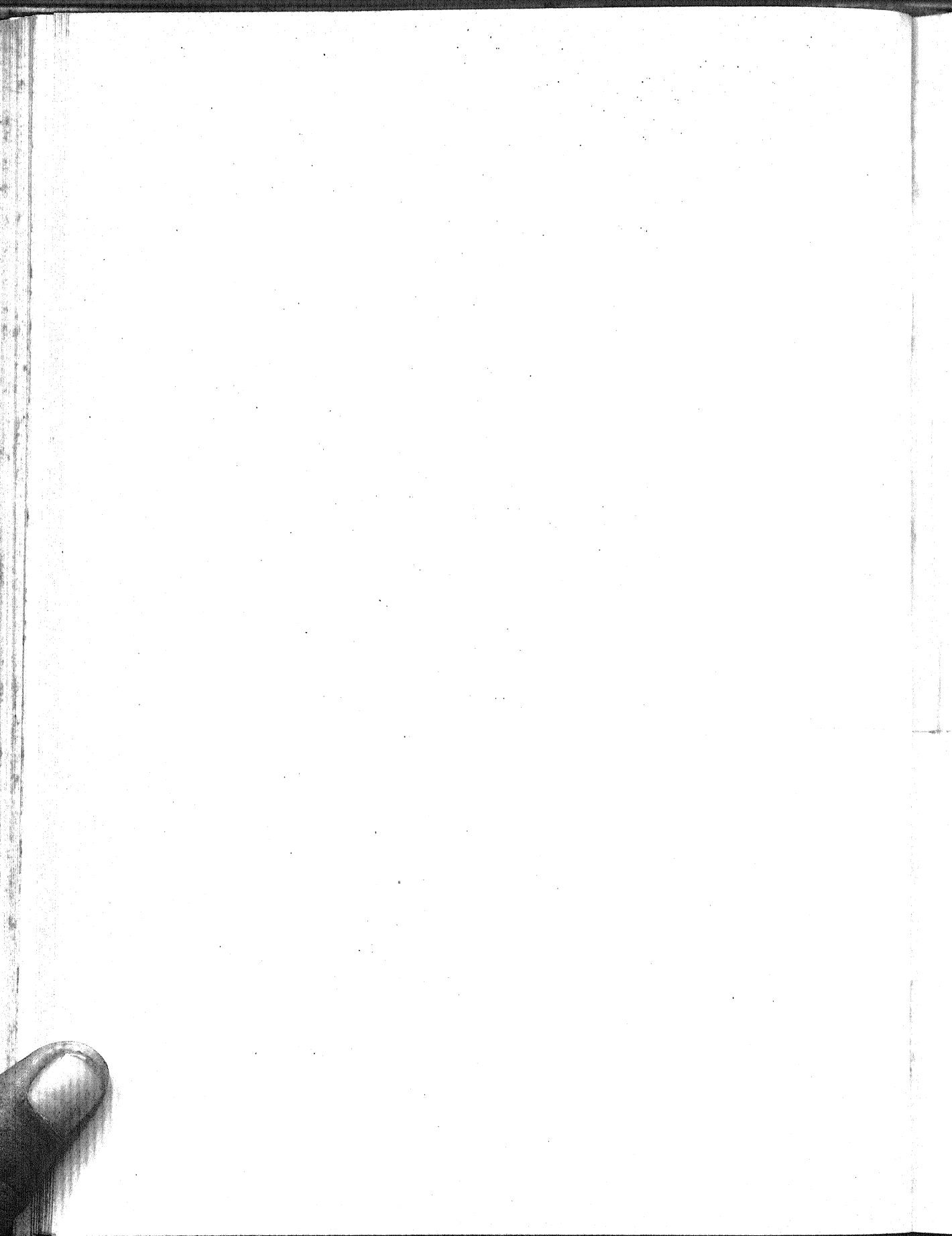
The author wishes to thank Prof. E. C. Dodds for his advice and encouragement, the Council of the Middlesex Hospital Medical School for the hospitality afforded and Mr M. E. H. Fitzgerald for his assistance with the practical work.

Part of the expense of this work was defrayed by a grant from the Thomas Smythe Hughes Medical Research Fund.

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OBITUARY NOTICE.

WALTER MORLEY FLETCHER.

(1873-1933.)

THE death of Sir Walter Fletcher, Secretary of the Medical Research Council, on June 7th, in the sixtieth year of his age and the nineteenth of his tenure of an office he had made great, removed with tragic unexpectedness a public servant who by sheer merit had raised himself to be the trusted captain and apologist of a great host of workers striving in their many different ways and for the most part in the seclusion of the laboratory for the advancement of scientific medicine.

These now mourn the loss of a friend. Medical research, as the late Sir Walter never ceased to emphasise in many a weighty and admirably drafted pronouncement, calls to its service the labour corps of many ancillary sciences and from none perhaps does it expect more help in the solution of its increasingly complicated problems than from Biochemistry.

To the members of the Biochemical Society, therefore, of which Fletcher was an original member, his loss is a particularly severe one, in that his appreciation of the part that Biochemistry has to play in the general advancement of medical science came from his own discipline and distinguished performance in physiological chemistry throughout a period of nearly twenty years.

Undoubtedly Fletcher would have called himself a physiologist first and foremost, but, so far as concerned the particular problem to which his laboratory life was dedicated, he had the clearest vision of its further illumination when helped on its way by modern biophysics and modern biochemistry—a vision that has more than materialised. The many appreciations of the late Sir Walter which have already appeared and their varied sources are eloquent testimony to the numerous links he had forged between the laboratory worker and the outside world of affairs. His task may be said, indeed, to have been to explain the day's work—the *ἔργα καὶ ἡμέραι*—of the laboratory to a non-scientific and not always too appreciative public, a public, however, which paid the piper and might even, if unwisely guided, call the tune. Fletcher was the conductor in fact of a great orchestra of many and strange instruments, and it was his business to harmonise and co-ordinate the tunes of each to the greater glory of medical progress. "Instruments," I venture to think, would have pleased him. It was a favourite notion of his that disciplines should be divided not according to their matter but according to their methods and that the latter alone had in them the seeds of universal value.

What then were Sir Walter's antecedents and how was it that he, a devoted man of science yet not unfamiliar with affairs, resigned his place in the orchestra to become its conductor when the call came to him just before the outbreak of the great war?

Fletcher began his academic career in 1891 when he entered Trinity College Cambridge. Three years later he graduated with first class honours in the Natural Science Tripos, and from that date for nearly twenty years the Cambridge School of Physiology was his workroom and Trinity College, of which he was elected

a Fellow in 1897 and to whose affairs he was devoted, his real home. His clinical training in medicine he took at Bart.'s and in 1900 he obtained his Cambridge M.B.

Throughout his whole Cambridge career Fletcher's immediate scientific interests were centred almost exclusively in the problem of muscle respiration, an important field of enquiry in which, at the outset of his work, little or no certain knowledge was available. Between 1898 and 1914 he contributed to the *Journal of Physiology* a valuable series of papers on this subject, including two in collaboration, respectively, with Hopkins and G. M. Brown. These papers definitely established Fletcher's reputation as a physiologist of distinction. His first contribution in 1898 on the survival respiration of muscle was a substantial one of nearly a hundred closely reasoned pages, and fully documented, the elaborate apparatus of which he made use for continuous estimations of CO_2 being a modification of that devised by F. F. Blackman for research on gaseous exchange in plant leaves. The work was carried out during his tenure of the Coutts-Trotter Studentship and was communicated to the 4th International Physiological Congress which met in Cambridge in August of that year.

The normal curve of CO_2 discharge from excised frog muscle was fully worked out and the effect thereon of various poisons and temperature changes. Of fundamental importance was his observation that in an excised muscle which has been made to contract, there was no accompanying increase in the rate of CO_2 discharge. Throughout this work Fletcher took pains to exclude from consideration late yields of CO_2 arising from putrefactive changes in the muscle.

In his second and third papers in 1902 another important stage was reached when, in investigating the influence of oxygen on the survival respiration of muscle, he showed that loss of irritability in a surviving muscle is greatly delayed by an abundant supply of oxygen.

In 1904 he recorded the fact that exposure of a fatigued muscle to an atmosphere of oxygen restored to it, in a marked degree, the osmotic properties of resting muscle.

In 1907, in collaboration with Hopkins, appeared a most important contribution to the biochemistry of muscle showing that contraction of excised muscle is regularly accompanied by an increase of lactic acid and that, if the fatigued muscle is placed in oxygen, the lactic acid already formed disappears. The paper contains also the technical details of a new colour reaction for lactic acid devised by Hopkins for this work.

Later papers in 1911, 1913 and 1914 (the last in collaboration with G. M. Brown) continue the discussion of lactic acid production particularly in connection with its alleged formation during autolysis and its formation in mammalian muscle.

Fletcher's work and that of his collaborator Hopkins laid the foundations on which was built the elaborate treatment on thermodynamic lines of muscular action by Hill and Meyerhof. An analysis of its main conclusions will be found in the Croonian Lecture before the Royal Society by Fletcher and Hopkins in 1915 (*Proc. Roy. Soc. Lond.* 1917, B 89, 444) while critical reviews by A. V. Hill of the relations existing between heat production in muscle and the underlying chemical processes will be found in the *Ergeb. d. Phys.* 1916, 15, 340 and in *Phys. Rev.* 1922, 2, 310.

Writing in 1923 (*Nature*, July 14th, Suppl.), A. V. Hill ascribed nearly all the recent advances in muscle physiology to the study of the phenomena of fatigue in muscle. Recovery from fatigue occurred apparently only in the presence of oxygen, while the lactic acid, which was found by Fletcher and

Hopkins to increase by exercise, was diminished or abolished by recovery in the presence of oxygen. Further work on the problem demanded a new technique for correlating the heat produced with the observed chemical phenomena. This was supplied by the delicate thermoelectric apparatus devised by Hill, and by its aid it has been possible to build up what is a completely new chapter in muscle physiology—the thermodynamics of muscular work. The mysterious lactic acid is now known from estimation of the heat evolved in contraction to be derived from glycogen. In recovery the lactic acid is rebuilt into the glycogen from which it was derived except for about one-sixth of it which is oxidised to provide energy for the restoration. The essential point is that contraction and discharge of lactic acid are anaerobic processes and that oxidation is concerned only with the phenomena of recuperation.

It has been said that Fletcher's soul was not fully and entirely satisfied by the strivings and triumphs of laboratory toil and that he sought a wider arena in which his intellectual versatility and his flair for managing might have full play. Possibly he felt that after 18 years' devotion to one single problem of high physiological importance, he could safely say that he had rounded it off quite satisfactorily according to his lights and that he might now pass on the burden of its further pursuit to workers of other disciplines, biophysics, molecular physics, enzyme chemistry and what not. The call came to him on July 1st, 1914, to take up the duties of Secretary of the Medical Research Committee founded in 1913 as part of the provisions of the National Insurance Act of 1911. From the wide scope of the Committee's reference, and its own views with regard to the extension of the term "Medical Research" as part of a national scheme, it was quite clear that, in the search for the ideal secretary, no ordinary person of narrow views and interests was likely to be successful in guiding its destiny. Fletcher proved himself no ordinary person.

This is how the Committee outlined their conception of research.

"The object of the research is the extension of new knowledge with the view of increasing our powers of preserving health and preventing or combating disease. But otherwise than that this is to be the guiding aim, the actual field of research is not limited and is to be wide enough to include, so far as may from time to time be found desirable, all researches bearing on health and disease whether or not such researches have any direct or immediate bearing on any particular disease or class of diseases provided that they are judged to be useful in promoting the attainment of the above object."

How well Fletcher and his Committee of changing personnel succeeded in translating into administrative practice the wide conception of research contained in this carefully drafted statement and how deftly he defended, when necessity arose, the more subtle and perhaps not too readily appreciated implications of its later phrases, are matters of history.

It is the history in fact of a highly fertile period of medical research in this country and the empire, which owes much of its success to Fletcher's gifts of co-ordination and the personal and friendly terms on which he invariably met the numerous workers and grantees in the vast organisation of which he held the threads.

This is not the place to record in detail the landmarks of Fletcher's 18 years' secretaryship—the pooling of the forces he wielded, at the very outset of his career, to meet the medical emergencies of war, the happily engineered emergence of the Medical Research Committee as the Medical Research Council with a new Charter and holding office with greater freedom and responsibility under the Privy Council, the centralisation at the National Institute, Hampstead, of

a permanent nucleus of experts in many fields of medical enquiry, and, as the years passed, the numerous opportunities seized by Fletcher to promote his one great ambition, to see the health of man and beast cared for and studied not only as a national and imperial charge but as the natural and fitting obligation of every right-thinking individual. One may fittingly refer here, however, to his many efforts in the cause of biochemical research and pay tribute to his many successful interventions in securing financial help for what are now active centres of research not only in biochemistry but also in nutritional studies. These centres have greatly enriched the resources at the Empire's disposal for attacking effectively the many problems that concern the health of its human and animal populations. Nutrition studies and all that these imply were very dear to Fletcher. Possibly the last reasoned article from his facile pen was a contribution to the new journal, *Nutrition Abstracts and Reviews* (1932), on the urgency of nutritional studies. There, in commending the new journal to its readers, he referred to the range and variety of interests served by advances in nutritional science and to the fact that no visible limits can be set to them. A perusal of the table of contents of any number of this new journal is indeed sufficient to indicate the far-reaching character of nutrition problems and goes far to establish a real truth underlying the adage "Der Mensch ist was er isst." Fletcher in his rôle of the complete advocate would have added "and beast too."

By his very position Fletcher could feel the pulse of medical progress at many points and generally with much acumen. So far as biochemical and nutritional science is concerned, its devotees owe him much for the sympathetic and always helpful interest he invariably took in their special problems.

Lastly, a glimpse of Fletcher in the midst of his daily office routine, interviewing friends and strangers, arranging committees and often presiding over them, translating draft reports, resolutions and preambles into the excellent English on which he justly prided himself and through it all, striving to secure action and effective action. It has amazed me to watch him preside, it might be, over a conference on poliomyelitis and to see him display his wonderful flair for ordered discussion and his real understanding of the subject in hand. His versatility was not that of the briefed barrister. Truly Fletcher's scientific discipline stood him well. Eighteen annual reports of his Council will inform the medical historian of the future what Fletcher did and what he strove to do to further the work and influence of an organisation which has abundantly justified its national character, but, without their aid, his memory will long remain green in the hearts of those who knew and loved and admired him.

J. C. G. L.

CLXXX. THE INFLUENCE OF THE GONADS ON PROTEIN METABOLISM.

III. (a) THE EFFECT OF INJECTIONS OF ANTERIOR PITUITARY EXTRACTS ON URINARY CREATI- NINE IN NORMAL AND CASTRATED RABBITS.

(b) THE TOLERANCE OF NORMAL AND CAS- TRATED ANIMALS TO INJECTED CREATINE.

BY ISIDORE SCHRIRE AND HARRY ZWARENSTEIN.

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(Received June 16th, 1933.)

THE evidence for the existence of an endocrine relationship between the testes and creatinine metabolism was presented in the first two papers of this series [Schrire and Zwarenstein, 1932, 1, 2]. This conclusion is supported by the observations of Vedel, Peuch and Lang [1926] who describe the case of a man of 56 who had been castrated and showed regression of the secondary sexual characters. His creatine and creatinine excretion was subsequently investigated. No creatine was excreted but the amount of creatinine in the urine was 2.8 g. per litre. According to Folin [1905] the concentration of creatinine in normal human urine varies from 0.61 to 2.0 g. per litre with an average of 1.08. In the second paper cited above it was suggested that the increased elimination of creatinine after castration was primarily due to hyperactivity of the anterior lobe of the pituitary, consequently injections of anterior lobe extracts should cause an increase in the excretion of creatinine. In this paper evidence is presented in support of the hypothesis. As a contribution to the more detailed analysis of the problem the effect of injections of creatine into normal and castrated animals was also investigated.

EXPERIMENTAL.

Details in regard to caging, feeding, collection and analysis of urine were given in the first paper of this series. In Table I the dates refer to the first of a number of 24-hour periods. The mean and the standard deviation of the daily

Table I. *Daily urinary creatinine, mg.*

Date	Normal (1)	Castrated (2)	Castrated (3)	Normal (4)	No. of 24-hour periods
A. Pre-periods:					
12. iv. 32	92 \pm 5.2	76 \pm 5.8	89 \pm 3.2	—	9
7. v. 32	93 \pm 5.5	93 \pm 3.5	91 \pm 2.4	—	5
17. vi. 32	97 \pm 3.1	87 \pm 4.3	79 \pm 1.0	86 \pm 4.1	4
Nos. 2 and 3 castrated 21. vi. 32.					
B. Post-castration periods:					
12. xii. 32	96 \pm 7.4	120 \pm 1.9	123 \pm 11.6	84 \pm 8.3	5
27. xii. 32	98 \pm 2.5	116 \pm 9.1	119 \pm 10.0	91 \pm 3.2	4
16. i. 33	95 \pm 3.7	111 \pm 2.6	122 \pm 5.2	98 \pm 7.0	4

creatinine output in mg. are given for each series of 24-hour periods. In all Tables rabbits Nos. 1 and 4 are the normal animals and Nos. 2 and 3 the castrated animals.

(a) INJECTION OF ANTERIOR LOBE EXTRACTS.

Parke-Davis antuitrin was used and also an extract of sheep anterior lobe prepared according to Bellerby's method [1933]. As a control an extract of brain prepared by the same method was injected. These were all injected subcutaneously.

Table II. *Daily urinary creatinine, mg.*

Date	Normal (1)	Castrated (2)	Castrated (3)	Normal (4)
16. i. 33	92	109	120	100
17. i. 33	96	109	114	92
18. i. 33	92	*109	126	*92
19. i. 33	101	115	127	109
20. i. 33	105	120	134	114
21. i. 33	100	109	120	93
22. i. 33	101	120	126	85
23. i. 33	100	†120	†126	†92
24. i. 33	92	120	126	112
25. i. 33	86	109	114	92
26. i. 33	100	120	120	92
27. i. 33	100	114	133	90

* On Jan. 18 No. 2 injected with 2 cc. brain extract, No. 4 with 2 cc. anterior lobe extract.

† On Jan. 23 No. 2 injected with 2 cc. anterior lobe extract, No. 3 with 2 cc. brain extract and No. 4 with 2 cc. antuitrin.

Table III. *Daily urinary creatinine, mg.*

Date	Normal (1)	Castrated (2)	Castrated (3)	Normal (4)
28. ii. 33	101	120	114	84
1. iii. 33	100	120	109	86
2. iii. 33	100	*109	*130	*90
3. iii. 33	104	120	123	110
4. iii. 33	98	110	109	80
5. iii. 33	96	109	121	96
6. iii. 33	93	†114	126	†80
7. iii. 33	100	109	120	106
8. iii. 33	104	120	130	109
9. iii. 33	92	109	120	92
10. iii. 33	93	109	130	88

* On March 2 Nos. 2, 3 and 4 each injected with 3 cc. antuitrin.

† On March 6 Nos. 2 and 4 each injected with 3 cc. antuitrin.

Table IV. *Daily urinary creatinine, mg.*

Date	Normal (1)	Castrated (2)	Castrated (3)	Normal (4)
10. iv. 33	96	120	133	100
11. iv. 33	96	126	140	88
12. iv. 33	*92	*120	*136	*96
13. iv. 33	125	134	140	96
14. iv. 33	100	122	125	96
15. iv. 33	100	133	126	96
16. iv. 33	†100	†126	133	†98
17. iv. 33	133	120	133	126
18. iv. 33	114	122	126	105
19. iv. 33	100	120	126	88
20. iv. 33	100	120	135	96

* On April 12 Nos. 1 and 3 each injected with 3 cc. anterior lobe extract; Nos. 2 and 4 each injected with 3 cc. brain extract.

† On April 16 Nos. 1, 2 and 4 each injected with 3.5 cc. anterior lobe extract.

The following conclusions can be drawn from the data presented above.

(a) Injection of antuitrin or of anterior lobe extract into normal animals caused an increased excretion of creatinine. The figures suggest that the increase bore a constant relation to the dosage. This is seen in the following table. The figures in brackets refer to the number of the animal. The average weight of rabbit No. 1 during the injection experiments detailed above was 2.74 kg. and of rabbit No. 4, 2.34 kg.

	Antuitrin		Anterior lobe extract		
Amount injected	2 cc.	3 cc.	2 cc.	3 cc.	3.5 cc.
Percentage increases	22 (4)	26 (4) 28 (4)	22 (4)	30 (1)	33 (1) 30 (4)

In all cases the rise occurred on the day following injection, persisted in a few cases during the next day and in all cases rapidly returned to the normal level.

(b) Injection of antuitrin or of anterior lobe extract had no effect on the high creatinine level in the castrated animals.

(c) Brain extract had no effect either in the normal or in the castrated animals.

Discussion.

Braier [1931] showed that there was a marked diminution in the creatinine excretion of hypophysectomised dogs. Relatively this effect comes on much sooner than the increased excretion of creatinine after castration, which indicates that the effect of castration is due primarily to the pituitary. The castration effect is retarded because it depends upon the gradual hypertrophy of the anterior lobe of the pituitary. The work of numerous investigators has established the fact that in many species castration leads to an increase in the weight of the anterior lobe [Moore and Price, 1932; Stein, 1933, who also give reviews of the literature]. Reese and McQueen-Williams [1932], have shown that the morphological changes in the anterior lobe of the male rat due to removal of the testes can be prevented by administration of extracts containing the active principle of the male gonads. The results of parabiotic experiments show that the hypertrophy is not due to defective utilisation and storage of hormones but involves increased production and secretion. Brauer [1929] found that anterior lobe hypertrophy after gonadectomy also occurs in rabbits although there seems to be some doubt whether females show any appreciable hypertrophy [Wolfe, 1932].

The depressing effect of the gonad secretions on the anterior lobe of the pituitary, only incompletely shown by the normal animal, is unmasked when the gonads are removed, and the effect of the removal of the inhibitory influence becomes more and more apparent as the anterior lobe gradually hypertrophies. This process is reflected in a gradual increase in the elimination of creatinine in the urine.

These facts together with those reported above afford strong evidence of a direct endocrine relationship between the anterior lobe of the pituitary and creatinine metabolism. The persistent increase in creatinine excretion which occurs as the result of castration implies the continued new formation of creatine and its transformation to creatinine. A steady level is reached after several months. This may be partly explained by the fact that hypertrophy of the anterior lobe reaches a maximum. Evans and Simpson [1929] noted in rats that the gonad-stimulating capacity of the pituitary slowly increased up to the 8th post-operative week, after which there was little increase, and Schenk [1927]

found that the cell-type changes in rats occur progressively until a level state is reached some time between the 4th and 7th post-operative months. On injection of anterior lobe extracts into castrated animals no further increase in the excretion of creatinine was obtained, and this indicates that cessation of pituitary hypertrophy is not the sole limiting factor in the production of a maximum steady level of creatinine output. It would be premature at this stage to attempt any detailed interpretation of the facts. Relevant data in regard to the creatine content of the muscles of normal and castrated animals and the effect of repeated daily injections of anterior lobe extracts are lacking.

(b) INJECTION OF CREATINE.

2-5 g. of creatine were injected subcutaneously into normal and castrated rabbits and the creatine excretions estimated in the urine. The animals used in this experiment were the same as those used in the injection experiments reported in the first part of this paper. In Tables V and VI the figures in brackets refer to the amounts of creatine excreted in mg. No creatine was excreted by the control animal or by the other animals on the days preceding the injection.

Table V. *Daily urinary creatine and creatinine, mg.*

Date	Normal (1)	Castrated (2)	Castrated (3)	Normal (4)
27. iii. 33	100	120	136	92
28. iii. 33	100	120	132	91
29. iii. 33	98	114	120	96
30. iii. 33	102	125	135	92
31. iii. 33	109 (291)	126 (474)	141 (659)	92
1. iv. 33	109 (21)	135 (65)	126 (74)	88
2. iv. 33	96 (10)	120 (21)	126 (25)	92
3. iv. 33	100	126 (24)	133 (12)	92
4. iv. 33	100	120 (17)	133 (5)	96
5. iv. 33	105	126	133 (17)	92
6. iv. 33	100	120	135	92
7. iv. 33	105	123	126	96

On March 30 Nos. 1, 2 and 3 each injected with 2 g. creatine.

Table VI. *Daily urinary creatine and creatinine, mg.*

Date	Normal (1)	Castrated (2)	Castrated (3)	Normal (4)
2. v. 33	100	126	133	96
3. v. 33	100 (1144)	127 (1739)	120 (1746)	100 (1300)
4. v. 33	100 (71)	120 (413)	126 (310)	100 (108)
5. v. 33	92 (31)	120 (40)	130 (41)	105 (19)
6. v. 33	91	118 (18)	135 (23)	93 (6)
7. v. 33	92	130 (20)	126 (25)	96
8. v. 33	100	133	130	100
9. v. 33	90	120	124	95

On May 2 Nos. 1, 2 and 3 each injected with 5 g. creatine; No. 4 injected with 3.8 g.

The results of Tables V and VI are summarised below:

	Normal	Cas- trated	Cas- trated	Normal	Cas- trated	Cas- trated	Normal
Creatine injected (g.)	2	2	2	5	5	5	3.8
Total creatine excreted (mg.)	322	601	792	1245	2230	2145	1433
Creatine retained (mg.)	1678	1399	1208	3755	2770	2865	2367
Percentage excreted	16.1	30.0	40.0	25.0	44.6	42.9	37.7

It is evident that normal and castrated animals behave differently towards injected creatine. In all cases the normal animal excreted less creatine and retained more than the castrated, *i.e.*, the normal animals have a high and the castrated a low tolerance to exogenous creatine. The castrated animal excreted a larger proportion of the injected creatine and also took longer to do so. The excretion of creatinine was not affected.

These results are in essential agreement with those of Remen [1932]. He found that intravenous injection of 500 mg. creatine into normal adult men between the ages of 20 and 53 is followed by no greater excretion of creatine in urine than before injection. Men of 70-90 years, a eunuch 43 years old and one man of 68 castrated for carcinoma excreted most of the injected creatine. One case of hypophyseal obesity, in which the normal excretion of creatine was high, reacted to injected creatine like normal adult men. The old men with failing sex function, the eunuch and the castrate thus showed a decreased tolerance typical of infants and children and, as shown above, of castrated rabbits. Remen concluded that the male sex glands have a regulatory influence on the metabolism of creatine. Lasch [1932] arrived at the same conclusion on the basis of similar experimental results. The evidence presented in the first part of this communication indicates that the difference between normal and castrated animals in regard to creatine-creatinine metabolism is due primarily to hyperactivity of the anterior lobe of the pituitary.

SUMMARY.

1. Injection of antuitrin or of anterior lobe extracts of the pituitary leads to an increased excretion of urinary creatinine in normal rabbits but has no effect on the high creatinine output of castrated animals.
2. The evidence presented affords evidence of a direct endocrine relationship between the anterior lobe of the pituitary and creatinine metabolism.
3. Normal rabbits show a high, and castrated animals a low, tolerance to subcutaneous injections of large amounts of creatine.

The authors wish to thank Messrs Parke, Davis and Co., London, for a supply of antuitrin.

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CLXXXI. THE BIOLOGICAL RELATION BETWEEN CAROTENE AND VITAMIN A.

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THE experiments of Moore [1929], since amply confirmed by numerous workers, clearly established the appearance in the liver of vitamin A following the oral administration of carotene. It has been rather generally assumed that the conversion occurs in that organ, but direct evidence of a satisfactory character has not yet been advanced. Wolff *et al.* [1930] removed a portion of the liver from rabbits which had been fed on a diet devoid of both carotene and the vitamins. Following an injection of carotene into the circulation the vitamin A content of the residual liver tissue, determined by the increase in the antimony trichloride colour reaction, showed a considerable increase at the end of 3 days.

The first claim to have brought about the conversion *in vitro* was made by Olcott and McCann [1931]. They incubated buffered suspensions of liver and of an enzyme preparation, "carotenase," made from that tissue with colloidal solutions of carotene dissolved in ethyl laurate. A loss of colour was observed and after 24 hours the unsaponifiable fraction extract prepared from the digests showed on spectroscopic examination a discrete absorption band at 3280 Å. The changes were not observed when the "carotenase" had been inactivated by heating.

The disappearance of the colour of the carotene in such incubation experiments, is, as Woolf and Moore [1932] point out, of no particular significance, but serious attention must be given to what appears to be clear evidence of the formation of a product with selective absorption at 3280 Å. and to the statement that no such substance is formed when the liver extracts have been heated.

One confirmation of the claims of Olcott and McCann has been reported by Parienti and Ralli [1932]. They detected an increased response to the antimony trichloride reaction after incubating colloidal solutions of carotene with minced dog liver, buffered at p_H 7.4. They also state that "carotenase" is inactivated by heating. Other investigators have failed to confirm these observations and the position at the moment is not satisfactory. Von Euler and Klusmann [1932] claimed that the absorption at 3280 Å. of cow-liver extracts was increased threefold after incubation with colloidal carotene, but in another communication [v. Euler, 1932] it is stated that "die Versuche von Klusmann mit kolloidalem Carotin zum Leberbrei in Boratpuffer konnten die Ergebnisse von Olcott und McCann nicht bestätigen."

In this laboratory Ahmad [1931] and Rea and Drummond [1932] failed to detect the production of a substance resembling vitamin A from carotene either by incubating with liver preparations *in vitro* or by perfusing into the portal circulation of surviving cats.

The failure of the latter technique was rather surprising and suggested that the tissues of a typical carnivore might not be well adapted for efficiently performing the conversion; it must be remembered that evidence is accumulating to show that the carotene molecule may be degraded or modified in a variety of ways in the animal organism.

In the work to be described the investigation has been extended to cover a typical example of the herbivores. In general the procedure has been similar to that already described.

EXPERIMENTAL.

Preliminary experiments to determine the distribution of vitamin A throughout the liver were made, and it soon became apparent that rather large variations were to be expected.

The liver samples were dried by intimate mixing with anhydrous sodium sulphate, and the fatty material was rapidly extracted in a continuous extractor by hot chloroform. The extracts were examined spectroscopically for pigments and for vitamin A. For this work a new form of photometer recently placed on the market by Adam Hilger Ltd., was used—the Notched Echelon Wedge Cell. This instrument possesses the advantage of enabling the estimations to be made with great rapidity; a matter of great importance when dealing with such labile substances as carotene and vitamin A which are easily decomposed photochemically.

The experiments on the distribution of vitamin A in the liver of rabbits were made on two different groups of animals. The first had been maintained for some 8 weeks on a diet of oats and bran in order to lower their tissue reserves of that substance. Those in the second group had received the usual stock laboratory rabbit diet containing ample supplies of green leaves.

In Tables I and II the figures referring to vitamin A concentration are expressed as the extinction coefficient ($\log_{10} \frac{\text{light transmitted}}{\text{light incident}}$ referred to 1 cm. of solution) at 3280 Å., at such a concentration that 1 g. of liver is contained in 1 cc. of the chloroform extract.

Table I.

Number	I	II	III	IV	V	VI	VII	VIII	IX
Weight, kg.	1.2	1.8	1.93	—	—	1.4	1.7	—	—
Diet	-A	-A	-A	—	—	Normal		—	—
Left anterior lobe:									
Weight, g.	21.9	3.7	7.6	6.4	43	8.65	21.55	26.4	25.0
Vitamin concentration	2.65	4.5	3.55	8.2	2.5	6.9	2.95	17.1	3.7
Fat concentration	—	—	—	—	0.0173	0.0337	0.0175	0.0264	0.0182
Caudate lobe:									
Weight, g.	3.1	—	10.9	11.2	30	6.6	17.3	25.1	27
Vitamin concentration	2.0	—	—	12.2	3.4	6.8	3.6	12.20	6.1
Fat concentration	—	—	—	—	0.0104	0.0279	0.0200	0.0344	0.0157
Left posterior lobe:									
Weight, g.	12.8	11.35	9.7	16.90	56.5	9.5	23.60	26.3	23.0
Vitamin concentration	2.2	4.75	3.55	7.1	2.1	7.6	3.05	11.8	5.9
Fat concentration	—	—	—	—	0.0069	0.025	0.0137	0.0351	0.0201
Right lobe:									
Weight, g.	9.6	10.35	12.8	22.6	52.5	9.9	20.25	28.8	26.0
Vitamin concentration	—	5.0	3.0	9.2	3.8	7.2	3.65	17.2	6.6
Fat concentration	—	—	—	—	0.0137	0.0265	0.0172	0.0324	0.0164
Maximum deviation from mean %	16	6	10	31	29	4.2	12	19	33.7

The results show that the distribution of vitamin A in these rabbit livers is far from being uniform. The differences are greater in the normally fed animals than in those which had been deprived of vitamin A in their diet. In most cases they are definitely greater than the experimental error, which we estimate at 5 %. Curiously, no sharp correlation was found between the distribution of fatty material extracted by chloroform and the vitamin, as might reasonably have been expected.

The injection experiments were made by the following procedure. A portion of one lobe of the liver was removed aseptically under ether anaesthesia and a sterile colloidal suspension of carotene in 5 % glucose solution was slowly injected directly into the portal vein. The carotene suspension was prepared according to the directions of Fodor and Schoenfeld [1931]. After the injection the incision was closed and the animal kept for a suitable period before removing the remainder of the liver for examination. These periods extended from 24 hours to 20 days and in most cases care was taken to examine separately the residual portion of the lobe from which the original sample had been removed.

Some of the results are summarised in Table II.

Table II.

Rabbit No.	I	II	III	IV	V
Weight, kg.	1.8	1.57	1.05	2.5	1.97
Duration of experiment, days	1	5	5	8	20
Liver, excised portion:					
Weight, g.	3.30	4.9	2.5	3.7	2.6
Vitamin content	4.8	11.0	2.02	12.0	3.2
Basal portion of same lobe:					
Weight, g.	—	—	4.1	7.64	9.10
Vitamin content	—	—	1.7	15.0	8.7
Residual portion:					
Weight, g.	45.0	65.0	49.8	40.30	54.3
Vitamin content	4.4	9.3	2.0	14.0	6.0
% Increase on mean	-8	-15.4	-8.4	+20.8	+130
Carotene:					
Injected, mg.	0.69	0.70	0.6	1.2	1.12
Recovered, mg.	0.35	0.52	0.42	0.9	0.80

These results provide no evidence of a definite increase in vitamin A in the liver until 8 days have elapsed after the injection of the carotene. Observation showed that the injected pigment is rapidly taken up by the liver after introduction into the circulation. This was also noted by van den Bergh *et al.* [1920] who found that carotene injected in the form of a colloidal suspension into the jugular vein of rabbits was soon removed from the blood and to a large extent was held by the liver.

Our experiments show that some part of the carotene is broken down or disappears from the liver, although the loss is not nearly so large as was observed in our earlier experiments with cats. We cannot exclude the possibility that formation of vitamin A had occurred in all our experiments, for it is conceivable that the depleted organism might immediately utilise the material for resumption of growth or repair of structure. If such were true a measurable increase in the amount present in the liver would, presumably, only be detected after the requirements of the body had been satisfied and storage had become possible.

The resumption of growth shown by the rabbits kept for the longer periods after operation, in which storage of new vitamin A was detected, rather suggests that this may be true (Fig. 1).

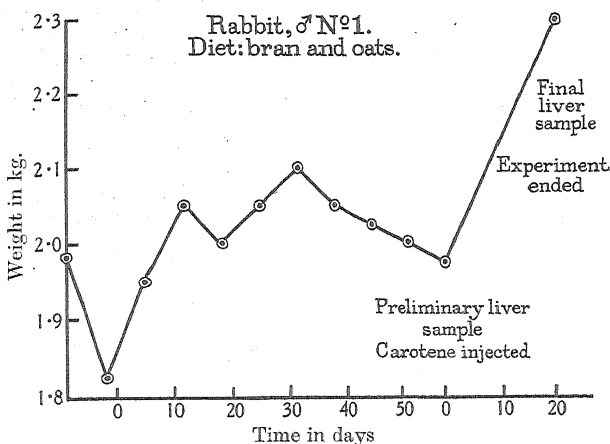


Fig. 1.

This work has been supplemented by a new series of experiments made to try and reproduce the conditions under which Olcott and McCann observed conversion of carotene into vitamin A *in vitro*. In every case the results were negative. Another method of attack was therefore devised, which it was thought would lead to success. The liver of a rabbit which had received an injection of colloidal carotene into the portal vein was removed after an interval sufficient to ensure that the pigment had been taken up in a normal manner from the circulation by the hepatic cells. Sterile preparations of the minced tissue suspended in phosphate buffer at p_{H} 7.3 were incubated at 37° for different periods. Afterwards the materials were extracted by chloroform and the extracts examined spectroscopically. Table III gives the results of four such experiments, but before stating the conclusion to which they lead us it is necessary to explain

Table III.

Number	Weight of liver, g.	Duration hours	Absorption at 3280 Å.	Absorption at 4800 Å.	Correction	Corrected absorption at 3280 Å.
I	2.21	0	5.5	—	—	—
	4.42	0	4.3	—	—	—
	2.17	48	5.75	—	—	—
	2.32	48	5.75	—	—	—
II	3.68	0	4.0	2.7	0.65	3.35
	3.77	24	3.95	2.7	0.9	3.05
	4.43	65	2.9	1.9	1.5	1.4
III	4.28	0	5.1	5.0	0.83	4.27
	0.72	0	5.1	5.0	0.83	4.27
	5.59	48	5.1	5.0	3.6	1.5
	4.16	120	5.1	2.0	2.0	3.1
	1.18	168	6.2	2.9	4	2.2
IV	4.80	0	17.7	1.8	0.3	17.4
	5.18	48	8.2	0.645	0.43	7.77
	7.14	120	7.0	0.645	0.645	6.38
	6.62	168	18.6	0.883	1.26	17.34
	6.53	168	18.6	0.645	0.92	17.68

the correction which it has been found necessary to apply in estimating the absorption in the region 3280 Å. We have confirmed the observations of McNicholas [1931], who found that solutions of carotene in ether and alcohol mixtures exposed to diffuse sunlight undergo oxidative breakdown in two stages. The initial changes are characterised by the appearance of an absorption band in the region of 3200 Å. with a corresponding decrease in the band at 2730 Å. The later stage is marked by a decrease in the colour of the solution and a widening of the ultra-violet bands which finally lose their entities.

A colloidal solution of carotene, such as we employed for our intravenous injections, when incubated at 37° in contact with air shows such changes. In Fig. 2 is represented the relation between the intensity of absorption at 4800 Å. and that at 3280 Å. as it changes during a period of 7 days; by 14 days the colour of the carotene had completely disappeared.

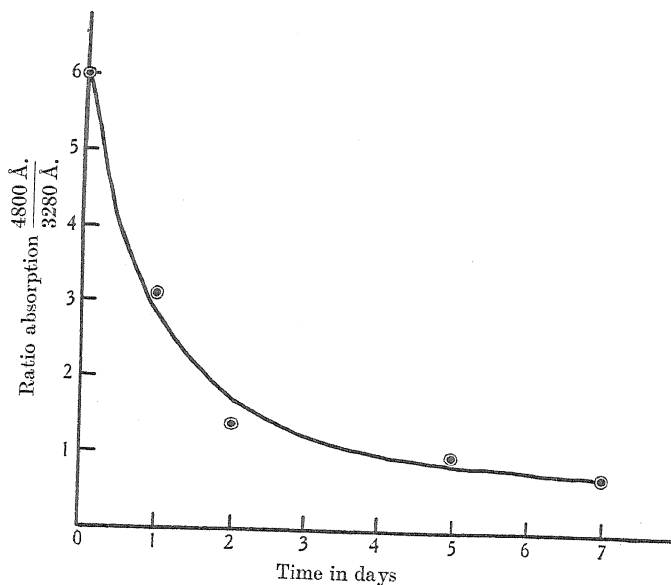


Fig. 2.

It is necessary, therefore, to apply a correction based on such a curve as that presented in Fig. 2 for all estimations of absorption at 3280 Å. made on preparations of carotene incubated in the presence of air. When such corrections are applied our results indicate that the incubated liver containing absorbed carotene shows at first a decreased absorption at 3280 Å. followed by a gradual increase. But a similar increase is shown by liver preparations to which carotene has not been added.

It is, however, impossible to apply a similar correction for the estimations made on the perfusion experiments described earlier in the paper because our observations show that a considerable proportion of the carotene may remain unchanged in the liver for periods as long as 20 days. In this organ the changes appear to be less rapid than in the solutions exposed to air.

SUMMARY.

After injecting carotene into the portal circulation of rabbits no increase in the vitamin A in the liver was observed, using a spectrophotometer method, until 8 days after the experiment. There is of course the possibility that formation of vitamin occurs within much shorter periods, but either that it is utilised and converted into another substance or that the amounts are too small to be detected by our technique. Further attempts to obtain conversion of carotene into vitamin A *in vitro* by incubation of liver tissues have met with no success.

In some of these experiments the conditions were made more "physiological" by permitting the liver cells to take up the pigments from the circulating blood before the minced preparation was incubated.

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CLXXXII. AN INVESTIGATION OF THE CAUSE OF RENAL HYPERTROPHY IN RATS FED ON A HIGH PROTEIN DIET.

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(Received July 3rd, 1933.)

THE problem of the enlargement of the kidneys of rats fed on diets rich in protein has, in recent years, been receiving considerable attention notably by Addis and the Mackays [1926, 1, 2] who have shown that the hypertrophy is proportional to the protein consumption and have elaborated a formula which expresses this relationship in terms of protein consumed and kidney weight per unit of surface area. The cause of this enlargement, however, still remains obscure. The excretion of urea naturally suggests itself as a causal factor. The feeding of urea, however, in amounts equivalent to what would be excreted on a high protein diet has been shown to produce a much smaller effect on the kidneys [Osborne *et al.*, 1926; Mackay, Mackay and Addis, 1931]. The effect of feeding proteins of different origin has however scarcely been investigated. Osborne *et al.* [1926] found that caseinogen, gliadin and beef residues all produced an enlargement of the kidneys but no actual numerical data on the basis worked out by the Mackays and Addis [1927; 1928] were elaborated. The object of the following experiments was an attempt to find out the cause of this hypertrophy.

Two possibilities suggest themselves. Do the kidneys in the rat form a depot for reserve protein, or is the hypertrophy a response to an increase in physiological function? It is possible in this connection that the kidneys are associated in some way with the intermediary metabolism of protein quite apart from the mere excretion of nitrogenous end-products.

EXPERIMENTAL.

A series of experiments was carried out to compare the following proteins and protein derivatives, and in addition the effect of an acid and alkaline diet—caseinogen, liver, gelatin, wheat gluten, glycine, glutamic acid, urea, NaH_2PO_4 and NaHCO_3 . The experimental procedure adopted in these experiments was practically the same as that employed by the Mackays [1927]. Standard albino rats 30 days old and obtained from the same source as far as each particular series of experiments is concerned were employed throughout. They were kept in cages open to the front with not more than 6 rats to a cage. The sexes were kept apart. The room in which they were kept was well lit and open to the south and was maintained fairly constant in temperature day and night. Water and food were allowed *ad lib.*, and the food consumption was measured each day. The rats were weighed about every third day. The duration of the experiments varied from 30 to 39 days in the different series. At the end of the experimental

period the rats were killed by chloroform and weighed. The abdomen was then opened, the kidneys removed, decapsulated, split in half, dried on filter-paper and weighed in a glass-stoppered bottle. The two kidneys were weighed together and the average taken as the weight of one. The surface area was calculated by Meeh's formula $S = BW^{\frac{2}{3}} \times K$. The constant $K = 11.36$ was that employed by the Mackays [1927] and found to be applicable to the rat by Carman and Mitchell [1926]. From the experimental data the following calculations have been made for each series. The average increase in body weight per rat daily; the average kidney weight per 100 cm.² body surface. The average daily protein consumption per 100 cm.²; the average increase in body weight per g. of protein consumed.

The first series of experiments was carried out with liver, gelatin and caseinogen. The object of testing liver was partly because it offers a protein differing considerably from caseinogen and partly because it is rich in extractives and nucleoproteins. Gelatin was chosen as a test protein because it has a low biological value and hence might be considered inadequate to build up any reserve tissue in the kidney. A further point of note is that gelatin is poor in tryptophan, cystine and tyrosine and according to certain workers [Newburgh and Marsh, 1925] these amino-acids are a contributing cause of the renal degeneration found in rats fed over a long period on a high protein diet. The diets employed are given in Table I and the results in Table II.

Table I. *Diets employed.*

(Figures represent parts per 100.)

Series	Dried liver	Casei-nogen	Gela-tin	Glu-ten	Gly-cine	Glu-tamic acid	Dex-trin	Lard	Urea	Salt mix-ture	Cod-liver oil	Dried yeast	Agar	NaHCO ₃	NaH ₂ PO ₄	Total protein %	Total Cals.
A	80	—	—	—	—	—	—	—	—	3	6	9	2	—	—	50.5	434
B	—	16	37	—	—	—	15	8	—	3	10	9	2	—	—	50.6	436
C	—	57	—	—	—	—	9	10	—	3	10	9	2	—	—	50.0	436
D	—	20	32	—	—	—	13	5	—	3	10	9	2	—	6	48.8	396
E	—	20	32	—	—	—	13	5	—	3	10	9	2	6	—	48.8	396
F	—	57	—	—	—	—	8	5	—	3	10	9	2	—	6	48.8	402
G	—	20	—	—	—	—	51	5	—	3	10	9	2	—	—	20.25	428
H	—	20	32	—	—	—	17	7	—	3	10	9	2	—	—	48.9	431
I	—	57	—	—	—	—	10	10	—	3	10	9	2	—	—	48.9	429
J	—	20	—	—	—	—	51	5	—	3	10	9	2	—	—	20.6	428
K	—	57	—	—	—	—	9	10	—	3	10	9	2	—	—	48.5	429
L	—	20	—	—	—	—	34.2	12	9.8	3	10	9	2	—	—	48.8*	426
M	—	20	32	—	—	—	17	7	—	3	10	9	2	—	—	48.5	431
N	—	20	—	—	—	—	56	—	—	3	10	9	2	—	—	20.2	402
O	—	20	—	26	—	—	30	—	—	3	10	9	2	—	—	42.7	402
P	—	20	—	—	10	—	42	4	—	3	10	9	2	—	—	31.4	403
Q	—	20	—	—	—	25	27	4	—	3	10	9	2	—	—	34.1	402

* Urea-N calculated as protein.

It will be noted that there is no difference in the kidney weights of groups A and C, *i.e.* those fed with liver and caseinogen respectively. The rats on the gelatin diet (B) showed however a considerable hypertrophy in comparison with groups A and C in spite of the fact that they actually consumed less protein. In order to ascertain if the differences between the groups are significant the standard deviation σ was calculated for each group and the probable error of the difference of the means calculated by the formula $0.6745 \sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}$ where n_1 and n_2 are the number of observations in each group and σ_1 and σ_2 the corresponding standard deviations. Where the ratio of the difference of the means to the probable error of the difference of the means is 3 or more the result is considered significant. The following are the data for series I.

A	σ	B	σ	C	σ
Males	0.03507	Males	0.00319	Males	0.02097
Females	0.01552	Females	0.0900	Females	0.02097
A and B males: Difference of means					0.066
Probable error of difference of means					0.0113
Ratio					5.84 : 1
A and B females: Difference of means					0.103
Probable error of difference of means					0.025
Ratio					4.12 : 1
B and C males: Difference of means					0.084
Probable error of difference of means					0.0082
Ratio					10.24 : 1
B and C females: Difference of means					0.099
Probable error of difference of means					0.025
Ratio					3.96 : 1

It will be seen that the difference is significant when the group fed with gelatin is compared with either of the groups fed with caseinogen or liver. It is possible that the increase in kidney weight of group B (gelatin) is due to the

Table II.

			Av. increase in body weight per rat per day, g.	Av. kidney weight per 100 cm. ² g.	Av. protein consumption per day per 100 cm. ² g.	Av. increase in body weight per rat per g. protein consumed
Series 1 31 days exp.	A	7	3.07	0.285	1.64	0.57
		7 + 0.01	2.10	0.239	1.57	0.52
	B	9	2.13	0.351	1.33	0.59
		6 + 0.01	1.76	0.342	1.58	0.46
	C	5	3.70	0.267	1.38	0.75
		12 + 0.01	2.03	0.243	1.39	0.51
Series 2 32 days exp.	D	6	1.12	0.403	1.50	0.32
		6 + 0.01	0.96	0.363	1.33	0.30
	E	6	2.0	0.395	1.43	0.46
		6 + 0.01	1.09	0.338	1.27	0.33
	F	6	3.05	0.324	1.41	0.62
		6 + 0.01	2.02	0.348	1.33	0.53
Series 3 39 days exp.	G	5	2.96	0.265	0.65	1.14
		6 + 0.01	1.67	0.227	0.67	0.81
	H	6	1.95	0.362	1.39	0.47
		6 + 0.01	1.34	0.340	1.39	0.38
	I	6	2.90	0.265	1.28	0.63
		6 + 0.01	1.60	0.237	1.38	0.40
Series 4 32 days exp.	J	6	2.01	0.218	0.67	1.07
		6 + 0.01	1.39	0.196	0.70	0.80
	K	6	1.73	0.221	1.14	0.59
		6 + 0.01	1.62	0.246	1.52	0.45
	L	6	1.89	0.236	1.44	0.52
		6 + 0.01	1.55	0.217	1.44	0.43
Series 5 32 days exp.	M	6	1.63	0.369	1.49	0.47
		6 + 0.01	1.20	0.336	1.69	0.32
	N	5	1.91	0.212	0.65	1.26
		5 + 0.01	1.57	0.199	0.64	1.16
	O	5	1.79	0.304	1.31	0.59
		5 + 0.01	1.31	0.270	1.31	0.47
	P	3	0.65	0.278	1.10	0.59
	Q	4	1.17	0.244	1.12	0.52

fact that although this group consumed less protein than groups A and C they actually catabolised more nitrogen. This assumption might be expected *a priori* since gelatin is almost incapable of promoting growth. This explanation would assume however that the hypertrophy is observed only when the protein is actually catabolised. In point of fact, as is seen from Table II, the increase in body weight per g. of protein consumed is approximately the same in groups A and B. It cannot however be inferred that the material added to the body in each group had the same nitrogen content.

In series 2 an attempt was made to repeat the work of Addis, Mackay and Mackay [1926, 1, 2] on the influence of adding acid or alkali to the diet. Nash and Benedict [1921] have shown that the kidney, in the dog at least, is the main if not the only organ concerned in the formation of ammonia and it was thought possible that the kidney hypertrophy might be secondary to an increased activity of this function following a high protein diet. The salts employed in the experiments were sodium dihydrogen phosphate and sodium bicarbonate. In experiments D and E (Table I, series 2) the diets were identical except that D was acid and E alkaline. The additional protein in D and E was gelatin. Mackay, Mackay and Addis [1926] have shown that phosphates themselves cause a considerable increase in kidney weight, and hence in groups D and E it would be possible to see if the hypertrophy of the kidneys in group D with gelatin and acid phosphate would be greater than in group E with gelatin and alkali. In short would there be a summation of those two factors in bringing about a hypertrophy? The experimental data are given in Table II, series 2.

It will be noted that there is scarcely any difference between groups D and E fed with gelatin and acid and alkali respectively. The difference in the kidney weights for the males is only 0.008 g. and for the females 0.025 g. per 100 cm.² body surface. Such differences are not significant. It thus appears that there is no summation of the effects of gelatin and phosphates when fed together in a diet. In group F fed with caseinogen *plus* acid phosphate the effect of the salt is quite marked. This is seen most clearly when groups C and F of series 1 and 2 are compared. The following are the statistical data.

D	σ	E	σ	F	σ
Males	0.03014	Males	0.0250	Males	0.0442
Females	0.07200	Females	0.0375	Females	0.0820
Series 2, D and F males:		Difference of means		0.079	
		Probable error of difference of means		0.0147	
		Ratio		5.37 : 1	
Series 2, E and F males:		Difference of means		0.071	
		Probable error of difference of means		0.139	
		Ratio		5.07 : 1	
Series 1 and 2, C and F males:		Difference of means		0.057	
		Probable error of difference of means		0.01371	
		Ratio		4.15 : 1	
Series 1 and 2, C and F females:		Difference of means		0.105	
		Probable error of difference of means		0.0214	
		Ratio		4.90 : 1	

Taking series 2 alone to begin with it will be noted that the difference between group D (gelatin and acid phosphate) and group F (caseinogen and acid phosphate) is significant in respect of the males. The kidney weights of the females of those two groups are however approximately the same. This contrast in the effect of caseinogen in series 2 as compared with series 1 must be attributed to the phosphates as the statistical data show when groups C and F of series 1 and 2

are compared above. The difference is significant for both sexes. The evidence from these experiments does not appear to favour the hypothesis that acidity or alkalinity of the diet, and hence ammonia formation by the kidney, is a factor in the renal hypertrophy. In series 3 an attempt was made to test the relative effect of superimposing caseinogen on the one hand and gelatin on the other on a diet low in caseinogen. The diets employed and the experimental data are given in Tables I and II, series 3 (G, H, I). Contrary to the findings of the Mackays [1927] there is no increase in the kidney weight when the protein of the diet is increased by the addition of extra caseinogen (groups G and I). In group H however where the increase in the protein of the diet is in the form of gelatin a hypertrophy of the kidneys is to be noted. The following are the statistical data.

G	σ	H	σ	I	σ
Males	0.03301	Males	0.02696	Males	0.02720
Females	0.03684	Females	0.03390	Females	0.02260
Series 3, G and H males:		Difference of means		0.097	
		Probable error of difference of means		0.0124	
		Ratio		7.82 : 1	
G and H females:		Difference of means		0.113	
		Probable error of difference of means		0.0137	
		Ratio		8.25 : 1	
H and I males:		Difference of means		0.097	
		Probable error of difference of means		0.0101	
		Ratio		9.63 : 1	
H and I females:		Difference of means		0.103	
		Probable error of difference of means		0.112	
		Ratio		9.19 : 1	
G and I males:		Difference of means		Nil	
G and I females:		Difference of means		0.010	

The increase in weight between groups G and H and H and I is very marked and merits special attention when comparing groups H and I which had practically the same percentage of protein in the diet and consumed almost the same quantity of protein per 100 cm.² body surface. Owing to the fact that additional caseinogen failed to cause a hypertrophy in the above series it was decided to repeat the experiments and in addition to compare the effect of urea and gelatin respectively with each other and with caseinogen. The diets employed and the experimental data are given in Tables I and II, series 4. Below are recorded the statistical results (J, K, L, and M).

The results in general confirm those of series 3. The increase in kidney weight on the high protein diet (K) as compared with the low (J) is significant with the female but not with the male group. The increase in kidney weight with the group fed with urea (L) as compared with the low protein (caseinogen) group (J) is significant but the differences are much less than those between groups J and M where the extra nitrogen is consumed in the form of gelatin. The difference is very marked in the case of groups L and M which consumed approximately the same amount of nitrogen—group L largely as urea and group M as gelatin. Assuming that the gelatin was all catabolised and excreted as urea, this experiment would confirm the fact that the excretion of urea is not the causal factor in the kidney hypertrophy. From the experiments in series 3 and 4 an estimate can be made of the increase in kidney weight per 100 cm.² body surface per

J	σ	K	σ	L	σ	M	σ
Males	0.0122	Males	0.0232	Males	0.0104	Males	0.0264
Females	0.0187	Females	0.0176	Females	0.0150	Females	0.0264
Series 4, J and M males:		Difference of means		0.151			
		Probable error of difference of means		0.008			
		Ratio		18.8 : 1			
J and M females:		Difference of means		0.140			
		Probable error of difference of means		0.008			
		Ratio		17.5 : 1			
J and K males:		Difference of means		0.003			
		Probable error of difference of means		0.0072			
		Ratio		0.041 : 1			
J and K females:		Difference of means		0.050			
		Probable error of difference of means		0.0067			
		Ratio		7.64 : 1			
J and L males:		Difference of means		0.018			
		Probable error of difference of means		0.0044			
		Ratio		4.09 : 1			
J and L females:		Difference of means		0.021			
		Probable error of difference of means		0.0062			
		Ratio		3.38 : 1			
L and M males:		Difference of means		0.133			
		Probable error of difference of means		0.0078			
		Ratio		17.02 : 1			
L and M females:		Difference of means		0.119			
		Probable error of difference of means		0.009			
		Ratio		13.22 : 1			

additional g. of protein consumed. In series 3 this has been calculated where the increase in protein consumed was derived from gelatin. The following are the values found.

Series 3, males	0.131 g. increase in kidney weight per 100 cm. ² per extra g. protein consumed per 100 cm. ²
females	0.156 g. increase in kidney weight per 100 cm. ² per extra g. protein consumed per 100 cm. ²
Series 4, males	0.184 g. increase in kidney weight per 100 cm. ² per extra g. protein consumed per 100 cm. ²
females	0.141 g. increase in kidney weight per 100 cm. ² per extra g. protein consumed per 100 cm. ²

These figures agree fairly closely with those obtained by the Mackays [1927] where the additional dietary protein was in the form of caseinogen.

In series 5 a cereal protein was tested and an attempt was made to find out which particular amino-acid or acids, if any, were responsible for the hypertrophy observed when feeding the whole protein. Wheat gluten, glycine and glutamic acid were selected as examples of cereal protein and amino-acids, glycine being chosen because gelatin contains a high percentage of this particular amino-acid and glutamic acid because of its high concentration in gluten. In the experiment with glycine 4 male rats were put on a diet containing 20 % of this substance; they tended to lose weight however and finally one died. The percentage of glycine was then reduced to 10 %. The animals in group Q however consumed a diet containing 26 % of glutamic acid quite readily. The diets and experimental data are given in Tables I and II (N, O, P and Q). It will be noted that both gluten and the amino-acids caused a hypertrophy of the kidneys. The following are the statistical data.

N	σ	O	σ	P	σ	Q	σ
Males	0.0275	Males	0.0356	Males	0.0328	Females	0.0091
Females	0.0250	Females	0.0317				

Series 5, N and O males:	Difference of means	0.094
	Probable error of difference of means	0.0135
	Ratio	6.96 : 1
N and O females:	Difference of means	0.070
	Probable error of difference of means	0.0122
	Ratio	5.37 : 1
N and P males:	Difference of means	0.066
	Probable error of difference of means	0.0152
	Ratio	4.34 : 1
N and Q females:	Difference of means	0.45
	Probable error of difference of means	0.0091
	Ratio	4.51 : 1

The differences in the weights of the kidneys per 100 cm.² per additional g. of protein (calculated from nitrogen consumed) consumed per 100 cm.² body surface have been calculated for the different groups, *e.g.* caseinogen-gluten where the additional protein refers to the last-mentioned.

N and O males (caseinogen-gluten) 0.139 g. increase in kidney weight per 100 cm.² per extra g. protein consumed per 100 cm.²

N and O females (caseinogen-gluten) 0.105 g. increase in kidney weight per 100 cm.² per extra g. protein consumed per 100 cm.²

N and P males (caseinogen-glycine) 0.146 g. increase in kidney weight per 100 cm.² per extra g. protein consumed per 100 cm.²

N and Q females (caseinogen-glutamic acid) 0.093 g. increase in kidney weight per 100 cm.² per extra g. protein consumed per 100 cm.²

O and P males ((caseinogen glycine)-(caseinogen gluten)) 0.123 g. increase in kidney weight per 100 cm.² per extra g. protein consumed per 100 cm.²

O and Q females ((caseinogen glutamic acid)-(caseinogen gluten)) 0.136 g. increase in kidney weight per 100 cm.² per extra g. protein consumed per 100 cm.²

If comparisons are drawn between animals of like sex it will be seen that the increase in kidney weight is proportional to the additional protein, *i.e.* N consumed. The addition of gluten to the diet causes an increase in the weight of the male kidneys of 0.139 g. per 100 cm.² body surface per additional g. of protein consumed. Comparing the females (N and O) on caseinogen and caseinogen *plus* gluten respectively with those on caseinogen and caseinogen *plus* glutamic acid respectively (N and Q) the differences are 0.105 g. and 0.093 g. per unit of surface per additional g. of protein consumed. Taking into consideration the nature of the experiment these results are fairly close, a difference of only 0.012 g. being observed between the groups. A similar comparison between the males of groups O and P (gluten and glycine respectively) against N as a standard shows a difference of only 0.007 g. between the groups. These results indicate that it is unlikely that any particular amino-acid is the factor concerned in the hypertrophy. It should be pointed out however that these differences are smaller than those obtained when gelatin is fed. In series 3 the figures giving the increase in kidney weight with gelatin as the protein superimposed are males 0.131 g. and females 0.141 g. per unit of surface area per additional g. of protein consumed. The corresponding figures of series 4 are males 0.184 g. and females 0.141 g. The averages of the two series—males 0.157;

females 0.148 g.—however, are approximately the same as those obtained by the Mackays [1927] where caseinogen at different levels was the protein fed. It will be recalled that in our experiments additional caseinogen caused a relatively small hypertrophy of the kidneys and in some cases none at all.

At this stage of the investigation however it cannot be said what is the cause of the hypertrophy. That it is a real increase in tissue substance and not a hydraemia has been shown in a recent paper by Mackay [1933], who found that the moisture content for all kidneys fell between 74 and 76 %. In confirmation of his findings the average water contents of the kidneys in series 5 exhibit the same consistency as shown below.

	H ₂ O %
Group N, males	75.1
females	73.7
Group O, males	76.5
females	76.4
Group P, males	76.0
Group Q, females	74.9

From the general trend of evidence however in the recent paper by Mackay [1933], which has just come to our notice, and from the results given in this paper, it appears that the cause of the hypertrophy is associated with some change common to all the amino-acids. Ammonia formation and deamination naturally suggest themselves. The first possibility appears unlikely in view of the experiments recorded here and by Addis and the Mackays [1926, 1, 2]. The question as to whether the kidney is capable of deaminating merits special attention in view of the work of Bollmann, Mann and Magath [1926], who have shown that, as far as the dog is concerned, the liver is the main if not the only organ capable of deaminating. Recent work by Holmes and Watchorn [1927] shows that the growing embryonic rat kidney produces ammonia and urea *in vitro*. Krebs [1932] has further shown that rat kidney *in vitro* is more active than rat liver in deamination and ammonia formation. Such results suggest strongly that, at least in the rat, the kidney may be concerned in deamination. In support of this hypothesis is the fact that the hypertrophy tends to be larger or more readily produced by the addition of gelatin than of caseinogen to the diet. It can be more or less assumed that ingested gelatin will be almost entirely catabolised while some of the caseinogen may readily be used for anabolic purposes. Experiments on the actual nitrogen excretion on the two diets would give information on this point. The application of these results to the treatment of renal disease can hardly be ventured as yet. Some of the kidneys of each group of series 3 were examined histologically and no abnormality was found as had already been noted by Osborne *et al.* [1926]. Newburgh and Curtis, however [1928], find definite signs of degeneration in the kidneys of rats fed over long periods on diets containing a high percentage of protein.

SUMMARY.

1. The increase in kidney weight of rats fed with a number of proteins and protein derivatives has been investigated.
2. Gelatin tends to produce a more marked increase in kidney weight than either caseinogen or liver.
3. Glycine, glutamic acid and gluten all produce an increase which is more or less proportional to the additional nitrogen consumed.

4. It is considered likely that the hypertrophy is associated with some stage in the intermediary metabolism of protein—probably deamination by the kidney.

The expenses of these investigations were defrayed by a grant from the Andrews Fund for which I express my indebtedness.

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CLXXXIII. THE OXIDASE AND DEHYDROGENASE SYSTEMS OF THE CRYSTALLINE STYLE OF MOLLUSCA.

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(Received June 15th, 1933.)

THE occurrence in the crystalline style of *Saxidomus giganteus*, *Paphia staminea* and *Mya arenaria* of substances having the general properties of enzymes and capable of oxidising some of the reagents commonly employed for the detection of oxidases was recorded in a previous paper [Berkeley, 1923]. Similar substances have since been found in the style of *Pecten maximus*, *Mytilus edulis*, *Ostrea edulis* [Yonge, 1925; 1926, 1]. *Ensis siliqua* [Yonge, 1925; Graham, 1931], *Schizothaerus nuttalli*, *Ostrea lurida*, *Ostrea virginica*, *Ostrea gigas*, *Bankia setacea* and *Cardium corbis* [Berkeley, 1933].

The observations that the oxidation of guaiacum could be brought about by an extract of the style of *Saxidomus giganteus* in the absence of free oxygen and that the style disappeared from animals kept in boiled water in sealed vessels and was regenerated on access of air led me to suggest that the style might play a part in the anaerobic respiration of lamellibranchs first recorded by Collip [1920]. Doubt has been thrown on the suggestion that there is any essential connection between the disappearance of the style and anaerobic respiration by Nelson [1925] and by Yonge [1925; 1926, 2]. Nelson contends that anaerobic conditions slow down the movement of the cilia of the style-sac and the activity of the style-secreting cells; the material of the style dissolved in the stomach is not replaced and the style therefore disappears quite apart from any possible part it might play in supplying oxygen. He also points out that no relation can be found between the sizes of the styles of molluscs living normally under very well and very poorly aerated conditions respectively. Yonge brings forward the same arguments and also shows that the disappearance of the style depends upon the relation between its p_H and that of the gastric fluid. The style has invariably the lower p_H , so that the portion of it which is exposed to the action of the stomach contents is normally dissolved, and its maintenance depends entirely on its continuous renewal by the secreting cells.

Whilst there can be no doubt of the validity of these contentions it still remained possible that an oxidation process brought about by the oxidase set free by the dissolution of the style might lead to the production during anaerobiosis of some or all of the CO_2 on the production of which the evidence for anaerobic respiration rests. This possibility could not be disposed of until the actual physiological reaction brought about by the activity of the oxidase was determined. The present paper deals with an attempt to determine this reaction.

The autoxidisable substance of the oxidase system.

The style of *Saxidomus giganteus* has been used throughout the experiments to be described. It was stated in my previous paper [Berkeley, 1923] that active oxidase preparations were obtained when styles of this clam were dried rapidly

at air temperature *in vacuo*. Further experience has led to a modification of this finding. It is true that the dried material, when freshly prepared, yields active aqueous extracts, but it has been found that it loses the power of so doing after keeping for a few weeks, even in a tightly closed container. The loss of activity is due to the destruction of the autoxidisable component of the oxidase system, and not to that of the peroxidase, since it is restored on addition of hydrogen peroxide to the extracts. It has further been found that, in order to obtain active oxidase preparations from undried styles taken from freshly-dug clams, it is essential that the anterior ends of the styles, which impinge on the gastric shield, be left intact. If these are removed preparations made from the remaining portion of the styles have little¹ or no oxidase activity. They are rendered active by addition of either hydrogen peroxide or of an extract of the anterior end of the style.

This observation led to the conclusion that the autoxidisable component of the oxidase system was in some way connected with the anterior end of the style. In clams which have recently been feeding this end of the style always carries a mass of finely ground food material consisting mainly of bottom-living diatoms. It was suspected that these might be the source of the autoxidisable substance, and experiments were therefore made with aqueous extracts of similar diatoms. A mass of these organisms was scraped off the glass sides of an aquarium tank. The material weighed about 2 g. after draining and drying as completely as possible with filter-paper, and consisted almost entirely of *Navicula* spp. It was ground with a little distilled water until microscopic examination showed that most of the cells were ruptured; more water was added and grinding continued for a further period. The material was then saturated with toluene, the volume made up to 15 cc. and allowed to stand for an hour or so. It was then centrifuged until microscopic examination showed practically no solid particles in suspension. Half a gram of crystalline styles dried *in vacuo* some weeks previously was finely ground and allowed to stand in 15 cc. of distilled water saturated with toluene for 17 hours at air temperature. At the end of this time most of the material had dissolved. The solution was centrifuged and tested for oxidase activity, with and without addition of an equal volume of the diatom extract, by the indophenol reaction and by means of *p*-phenylenediamine, α -naphthol and pyrogallol. The diatom extract, without addition of the style extract, was tested simultaneously. The mixed extracts gave the most rapid and strongest reactions with each reagent. The style extract alone gave a slight indophenol reaction, but did not oxidise the other reagents. The diatom extract alone showed some slight activity towards pyrogallol, but was practically without action on the other reagents. The rather dark yellow-green colour of the diatom extract prevented the satisfactory use of guaiacum as a reagent for these tests owing to the difficulty of detecting the development of blue. A parallel series of tests was carried out, however, using an extract of plankton diatoms (obtained by towing in the open sea and consisting mainly of *Chaetocerus* spp.) made in the same manner. The reactions obtained were similar and, in this case, the guaiacum reaction could be applied since the diatom extract was only slightly coloured. It confirmed the other tests; the style extract alone being quite inactive, the diatom extract very slightly so, and the mixture giving a strong and rapid reaction.

¹ Removal of the anterior end of the styles eliminates the greater part, but not all, of the diatom food-material. Some inevitably remains both on and in the style. This probably accounts for the fact that extracts of styles which have had their anterior ends removed sometimes show slight oxidase activity.

It was evident that the autoxidisable component of the oxidase system of the style was contained in the diatoms adherent to it. A confirmation of this was obtained from clams which had been kept in the sea in a floating cage during a period of the year when the surface water contained little or no plankton. As a consequence the clams had been unable to feed and the anterior ends of their styles were quite free from diatoms. An extract of these styles was entirely without oxidase activity, but it was established by addition of either an extract of diatoms or of hydrogen peroxide.

The nature of the diatom constituent involved has yet to be determined. It does not seem to be as unstable as was to be anticipated from the rather rapid loss of oxidase activity by the dried styles, so long as the diatoms remain intact. Some of the bottom diatom material used in the previous experiments was dried *in vacuo* and kept for some weeks. It was then ground and extracted and a series of tests carried out with the extract to determine its power of inducing oxidase activity in an inactive style extract relatively to that of an extract of undried diatoms. The two diatom extracts were made as nearly as possible of the same concentration. The dry material led to only slightly less oxidising activity than the wet, indicating that little destruction of the autoxidisable substance had resulted from drying and storage. The destruction of the autoxidisable substance is presumably due to oxidation by atmospheric oxygen, and it is probable that this occurs more rapidly in the material occurring naturally on the style than in the intact diatoms owing to the fine state of division to which it has been reduced. Filtration of diatom extracts removes the autoxidisable substance almost entirely.

Since it was not always possible to obtain live clams as they were required, extracts of dried styles, which were inactive without addition of the autoxidisable substance, and of fresh diatoms, made as described above, have been used for a large part of this work. Moreover, this course had the advantages that solutions of greater oxidising activity could be made than were obtained by using fresh styles and that the parts played in their joint action by style extract and diatom extract respectively could be separately studied.

Selection of substrate.

Since all molluscs which have a crystalline style are herbivores [Yonge, 1930], and the style is mainly concerned in grinding food the organic portion of which consists largely of polysaccharides and submitting it to preliminary digestion, it was anticipated that the substance acted upon by the oxidase was a carbohydrate. It has long been known that the style contains an amylolytic enzyme, but there is no evidence that the style alone can carry the hydrolysis of starch to the formation of glucose, indeed evidence to the contrary has been brought forward by Yonge [1926, 1, 2], who showed that maltase is not present in the style of *Ostrea edulis*, though it occurs in the digestive diverticula. The production of glucose certainly occurs after the ground food mixed with style material leaves the stomach, and it is unlikely that any oxidative process would take place until this stage of hydrolysis had been reached. Accordingly it was decided to experiment in the first place with glucose as substrate.

Acid formation.

All attempts to detect the formation of an acid substance by the action of style extract on glucose failed. Freshly dug clams were used for these experiments and care was taken to retain the anterior portion, carrying the diatom

mass, in dissecting out the styles. These were dissolved in distilled water, the p_H of which had been adjusted at 7.0, saturated with toluene, using 1 style (weighing on average 0.2 g.) per 1 cc. of water. Solution was almost complete after standing for a period of 12 hours at air temperature, at the end of which the small amount of undissolved material was removed by centrifuging. Solutions thus prepared reacted strongly with oxidase reagents. Their p_H ranged from 6.2 to 6.0. The p_H of the style of *Saxidomus giganteus* is thus higher than that recorded for other lamellibranchs [Yonge, 1931]. Quantities varying from 1.25 to 5 % of pure glucose were added to tubes each containing 5 cc. of such style solutions and bromocresol purple as indicator. A set of tubes was allowed to stand at air temperature and another in a bath kept at 30° to 35°. No change of p_H could be observed in any of the tubes during periods up to 3 days. It seemed probable that the large amount of protein present in the style extracts exerted so strong a buffering action that an actual development of acid produced no corresponding alteration in p_H . It was found, however, that addition of one drop of a 0.01 % solution of formic acid to any one of the tubes produced a recognisable alteration in the colour of the indicator. It was concluded, therefore, that if the glucose were undergoing oxidation, the process did not result in the production of an acid.

Production of a substance giving the tests for glucosone.

Since glucosone occurs in addition to acids amongst the products of the mild oxidation of glucose an attempt was made to identify this substance as a reaction product of the style extract on glucose. For this purpose a test was necessary which would detect glucosone in small concentration in the presence of relatively large amounts of glucose. A solution containing glucosone was prepared from pure glucose by oxidation with copper acetate, following the method of Evans *et al.* [1928]. Glucosone was not isolated. The solution, after removal of copper, was used direct for the purpose of standardising the tests, and calculations of concentration were made on the assumption that oxidation to glucosone had occurred at the rate indicated by Evans *et al.* under the experimental conditions laid down by them.

A solution of 0.5 g. of *m*-nitrophenylhydrazine in 30 cc. 2N HCl was found a satisfactory reagent. Equal volumes of the reagent and of the solution under test are mixed and allowed to stand at 37°. A 0.03 % solution of glucosone produces a precipitate in half an hour. A 5 % solution of glucose is without action in that time. Dixon and Harrison [1932] used a 2 : 4-dinitrophenylhydrazine reagent made up in the same way for the detection of glucosone. The reagents seem to be of about equal delicacy.

It will be shown later that positive reactions were obtained with this test in application to solutions of crystalline style containing glucose, but, since it was not found possible to obtain sufficient of the reaction product to identify it by melting-point or other quantitative means, it was considered advisable to supplement the test by the following two others.

I. *Reduction to fructose and detection by the Seliwanoff test.* Glucosone on reduction by zinc dust and acetic acid yields fructose which can be detected in very dilute solution by means of the Seliwanoff test. Since glucose in relatively concentrated solution reacts to some extent it is necessary to remove most of it from the solutions to be tested. This can be accomplished by oxidation with bromine water, which leaves fructose practically unaltered. The procedure employed is as follows. 1 g. Zn dust and 0.5 cc. of glacial acetic acid are added to 5 cc. of the solution under test and the mixture is heated in a loosely-corked

test-tube standing in boiling water for 2 hours. After cooling the undissolved Zn is filtered off and 20 cc. of bromine water (saturated at air temperature) are added. The mixture is allowed to stand at air temperature in a closed vessel for at least 4 days, after which it is evaporated on a water-bath to 5 cc. An equal volume of 25 % HCl is added to this and a trace of resorcinol and the solution is heated in boiling water for a minute and quickly cooled. The presence of 0.02 % of glucosone produces a quite definite red colour. A 5 % solution of glucose gives none.

II. *Schiff's reaction*. Dixon and Harrison [1932] mention that glucosone gives this reaction. In the experiments to be described equal volumes of the solution under test and of the reagent were taken. The presence of 0.04 % of glucosone is easily detected. Glucose is entirely without action.

The presence of compounds other than glucosone might be indicated by either of these tests, but there would seem to be no other substance resulting from the oxidation of glucose which gives all three. The occurrence of positive reactions in all three cases affords, therefore, strong presumptive evidence of its presence and its formation has been assumed when this has occurred, though it is realised that the finding needs final confirmation by quantitative means.

Before applying the tests it was essential to remove all protein from the solution. This was accomplished by dialysing through cellophane. It was necessary to concentrate the dialysate before testing. Dixon and Harrison [1932] show that solutions of glucosone may be concentrated *in vacuo* in acid solution without appreciable loss. This has been confirmed.

A preliminary experiment carried out with mixed extracts of wet bottom diatoms and dried styles indicated that glucosone was produced. To 10 cc. of an extract of the diatoms made as described in a previous paragraph 0.25 g. of finely ground dried style and 0.5 g. glucose were added, the liquid was saturated with toluene and the mixture shaken at intervals until the style material was dissolved. It was then put into a wide-bottomed flask, in order to expose as large a surface to the air as possible and allowed to stand at air temperature for 48 hours. At the end of this time it was transferred to a small cellophane bag and dialysed against about 50 cc. of distilled water saturated with toluene. The water was changed at 12-hour intervals until, on testing with Fehling's solution, only a slight reducing action was obtained. The united dialysates were concentrated to 10 cc. *in vacuo* at 50–60°, after addition of 0.5 cc. of glacial acetic acid.

The three above-mentioned tests applied to 2 cc. samples of this concentrate gave in each case a positive reaction for glucosone. This has been repeated several times using extracts of both bottom and plankton diatoms, and similar results have been obtained using solutions of undried styles extracted from freshly-dug clams with care to retain the anterior ends carrying the diatoms, but, in the latter case, the glucosone reactions were never so strong as in those from solutions of the same concentration of dried style in diatom extract.

In order to determine to what extent the production of glucosone was to be attributed to the activity of each component of the mixtures, series of experiments were carried out in which each constituent was omitted in turn. The concentrations of the extracts and of glucose were the same as those in the experiments described above. The results obtained are summarised in Table I.

It is evident that the oxidation of glucose to glucosone by the combined extracts of style and diatoms depends upon the presence of the latter. The extract of diatoms alone can bring it about to only a small extent. The reaction proceeds in this respect in a manner precisely analogous to the oxidation of

Table I.

Mixture	<i>m</i> -Nitrophenylhydrazine	Fructose test	Schiff test
Style, bottom diatoms and glucose	Precipitate in 15 mins.	Strong colour	Coloration in 2 mins.
Style, plankton diatoms and glucose	Precipitate in 30 mins.	Strong colour	Coloration in 5 mins.
Style and bottom diatoms	Trace of precipitate in 30 mins.	Very slight reaction	Faint coloration in 6 mins.
Bottom diatoms and glucose	Slight precipitate in 30 mins.	Distinct coloration	Faint colour in 2 mins.
Plankton diatoms and glucose	Slight precipitate in 30 mins.	Fair coloration	Faint colour in 5 mins.
Style and glucose	No reaction	Trace of colour	No reaction

guaiacum or pyrogallol. In the absence of added glucose the glucosone reactions are very slight. Doubtless the trace which is found may be attributed to the glucose produced by the hydrolysis of carbohydrate in the diatom extract.

Presence of a dehydrogenase.

The nature of the oxidation of glucose brought about by the style with its added diatoms, consisting as it does of the removal of hydrogen from the glucose molecule, suggested that a dehydrogenase might be concerned.

An experiment was carried out with a mixture of extracts of bottom diatoms and dried style, made as described above, and arranged to detect the presence of such an enzyme. The liquid was centrifuged as soon as solution of the style material was complete, 5 % of glucose and 1 cc. of a 1/10,000 solution of methylene blue were added, and, after removal of a small sample for colour comparison, the mixture was immediately put into a Van Slyke [1917] CO₂ apparatus and pumped free from dissolved air. After standing *in vacuo* in this apparatus for 24 hours some decoloration of the methylene blue was evident by comparison with the sample removed before exhaustion, but it was difficult to estimate its degree because the blue was obscured by the yellow-green of the diatom pigment. After standing another 24 hours, during which the green colour of the solution became appreciably yellower, it was removed from the apparatus and heated to 98° for 2 minutes to inactivate the enzymes and so avoid risk of oxidation proceeding under the action of the oxidase during dialysis. After cooling the liquid was dialysed and the dialysate concentrated and tested as previously described. *m*-Nitrophenylhydrazine gave a definitely positive reaction for glucosone in 15 minutes and its presence was confirmed by the Schiff test.

Although it was not probable that the glucosone formed in this experiment was due to oxidation under the influence of the oxidase of glucose derived from the diatoms during the dissolution of the style material, since, as has been shown above, only traces are produced in this way, it seemed advisable to eliminate the possibility. The experiment was therefore repeated using plankton diatoms, which give a lightly coloured extract, in which decoloration of methylene blue can be easily followed, and an extract of dry style in water. The glucose was dissolved in the style extract and the diatom extract mixed with it immediately before addition of the methylene blue and exhaustion of air. After standing 6 hours *in vacuo* considerable decoloration had taken place; after 24 hours it was almost complete. 0.5 cc. more methylene blue solution was then added and, after re-exhausting, the solution was allowed to stand a further 24 hours during which further decoloration occurred. Strong reactions for glucosone were given by the concentrated dialysate obtained from this experiment.

Finally a similar experiment was carried out with a solution of undried styles, with their normal complement of attached diatoms, in water (about 3 g. styles to 15 cc. water). Only slight decoloration of the methylene blue took place, and all the glucosone tests were weak though definitely positive.

It was evident therefore that the combined extracts of style and diatoms contained a substance, or substances, which could reduce methylene blue and lead to the production of glucosone from glucose *in vacuo*. It remained to be determined whether these two reactions were connected and how far each constituent of the mixture was essential to them. To this end a series of experiments was carried out in which each constituent of the complete mixture was eliminated in turn. The experiments were maintained *in vacuo* for periods of 48 hours. Both bottom and plankton diatoms were used, but, since the results confirmed one another almost completely, they are not reported separately (Table II).

Table II.

Mixture	Decoloration of methylene blue	<i>m</i> -Nitrophenyl-hydrazine	Fructose test	Schiff test
Style, diatoms, glucose and methylene blue	Almost complete	Precipitate in 15 mins.	Strong colour	Colour in 6 mins.
Diatoms, glucose and methylene blue	Slight	Trace of precipitate in 30 mins.	Slight colour	Slight colour in 15 mins.
Style, glucose and methylene blue	Slight	Slight turbidity	Slight colour	No reaction
Style, diatoms and methylene blue	None	—	—	—
Glucose and methylene blue	Very slight	No reaction	Very slight colour	No reaction
Style, diatoms and glucose	—	Slight turbidity	Trace of colour	Very slight colour in 15 mins.

It is apparent from these results that the decoloration of methylene blue under the combined action of style and diatoms depends upon the presence of glucose; without it no decoloration occurs. Also that in the absence of methylene blue practically no formation of glucosone occurs, so that methylene blue is evidently essential as hydrogen acceptor for the dehydrogenation of glucose to proceed. It seems reasonable therefore to look upon the two reactions as connected by a dehydrogenase system transferring hydrogen from glucose to methylene blue. The full activity of this dehydrogenase system depends upon the presence of a diatom constituent. The decoloration of methylene blue and the glucosone reactions are all more pronounced in the case of the mixed extracts than in that of either alone. This diatom constituent, unlike that which functions as the autoxidisable component of the oxidase system, becomes inactive in dried diatoms which have been kept for some weeks. An extract of such dried diatoms in which dried styles and glucose had been dissolved did not decolorise methylene blue appreciably faster or more completely than a solution of the same concentration of styles and glucose in water, and the mixture led to only very slight glucosone reactions. Mann [1932] has shown that a hydrogen activator plays a part in the oxidation of glucose by the glucose dehydrogenase isolated by Harrison [1931] from liver. It is probable that such an activator is present in diatoms, but the evidence available does not exclude the possibility that the dehydrogenase is contained in the diatoms and an activator in the style. This point has yet to be investigated.

DISCUSSION.

The observations that glucose is oxidised to glucosone by the activity of the crystalline style in contact with air under the influence of an oxidase system and also out of contact with air under that of a dehydrogenase system seem to demand a common explanation. The most probable one appears to be that the production of glucosone in solutions in contact with air is due to the activity of the dehydrogenase system and that the oxidase system serves merely as hydrogen acceptor to promote this activity, but proof that this is the case awaits the separation of the various substances concerned. That an oxidase system and a dehydrogenase system can thus interact has been shown by Harrison [1931] who was able to promote the oxidation of glucose in contact with air under the influence of his glucose dehydrogenase by means of an oxidase isolated from heart muscle. In this case cytochrome was necessary to serve as carrier between the two activating systems and, if the analogy be complete, some substance performing this function is to be looked for in the crystalline style or its accompanying diatoms.

It is clear that Harrison's glucose dehydrogenase differs essentially from the dehydrogenase of the crystalline style inasmuch as the one leads to the production of gluconic acid, the other to that of glucosone.

The only other enzymes bringing about the oxidation of glucose which have been described appear to be that of Müller [1929] isolated from *Aspergillus niger* and that which occurs in certain bacteria. The former behaves as an oxidase and produces gluconic acid. The latter is inactivated by toluene [Quastel and Wooldridge, 1927]. Both, therefore, differ from the dehydrogenase of the crystalline style.

This appears to be the first time that the production of glucosone as the result of animal, or partly animal, physiological activity has been described; it is indeed the only record of its occurrence as a biological product except that of Walker [1932] who detected it in a glucose medium in which a mould of the *flavus-oryzae* group of *Aspergilli* had been grown.

If the formation of glucosone from glucose is the only reaction brought about by the oxidising activity of the crystalline style, as seems to be the case, the suggestion that the CO_2 produced by clams during anaerobiosis might be attributed to that activity must be abandoned, since CO_2 is not a product of the reaction.

It is realised that the results which have been recorded in this paper are of a preliminary nature. Future work will be directed toward the separation of the several substances concerned in the reactions which have been described, the determination of the conditions governing their interactions, the quantitative identification of the product or products, and the effect of substituting other substrates for glucose.

SUMMARY.

1. The oxidase system of the crystalline style of *Saxidomus giganteus* depends for its activity on at least two components, a peroxidase contained in the style itself and an autoxidisable substance contained in the diatoms constituting the food of the animals.
2. No acid is formed by the action of the oxidase system on glucose.
3. Glucosone appears to be formed by the action, but this needs quantitative confirmation.

4. A dehydrogenase system also occurs whose activity also depends on the joint action of a substance contained in the style and another contained in the diatom food material.
5. Glucosone also appears to be formed by the action of the dehydrogenase system on glucose in the presence of methylene blue *in vacuo*.
6. It is suggested that the oxidase system serves as hydrogen acceptor to promote the action of the dehydrogenase system.

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CLXXXIV. THE KERNEL-FATS OF SOME MEMBERS OF THE PALMAE: *ACROCOMIA SCLEROCARPA* MART. (GRU-GRU PALM), *MANICARIA SACCIFERA* GAERTN., *ASTROCARYUM TUCUMA* MART., *MAXIMILIANA CARIBAEA* GRISEB., *ATTALEA EXCELSA* MART. (PALLIA PALM), AND *COCOS NUCIFERA* LINN. (COCONUT).

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(Received July 12th, 1933.)

Up to the present only five examples of the kernel-fats of this important family have been examined by the methods now available for the quantitative estimation of the component fatty acids [Armstrong *et al.*, 1925; Taylor and Clarke, 1927; Hilditch and Vidyarthi, 1928; Collin and Hilditch, 1928; Saraiva, 1929; Heiduschka and Agsten, 1930], and, of these, two only have been submitted to oxidation with potassium permanganate, whereby a general idea of the glyceride composition may be obtained. In the present paper the relevant data for six more seed-fats of this family are communicated. Of these, five are new, in the sense that they have not hitherto undergone detailed analysis, and one, a commercial specimen of coconut oil, is of interest as a useful addition to the example already reported upon in an earlier paper [Collin and Hilditch, 1928].

Of the five new fats, those from *Acrocomia sclerocarpa* Mart., *Manicaria saccifera* Gaertn., and *Astrocaryum Tucuma* Mart., were available in sufficient quantity for a fatty acid analysis of the normal standard of accuracy and for a determination of their content of fully-saturated glycerides. The amounts of fat available from the kernels of *Maximiliana caribaea* Griseb. and *Attalea excelsa* Mart. were so small that it was only possible to make a very rough estimate of their component fatty acids. In the case of *Maximiliana caribaea* the fatty acid mixture was subjected to a single fractionation, and its general composition was then deduced from the analytical constants of the primary fractions, in the absence of the systematic refractionation which is strictly necessary when dealing with the complex mixture of acids characteristic of the Palmae seed-fats. In the other case, the kernel-fat of *Attalea excelsa*, the primary fractionation was controlled so as to correspond as closely as possible, in size of fraction and range of boiling-point, with an average primary fractionation of a Palmae kernel-fat, and by comparison of the two sets of analytical data a general idea of the composition of its component acids was obtained.

To guard against any possibility of loss of low-boiling esters during the vacuum distillation, a single-surfaced water-condenser was inserted between the Willstätter bulb and the Perkin triangle during the primary fractionation

and the first two refractionations. Apart from this alteration, both the ester fractionations and the acetone-permanganate oxidations follow the procedures adopted in the examination of coconut and palm kernel-fats [Collin and Hilditch, 1928].

COMPOSITION OF THE FATTY ACID MIXTURES.

The tendency of the various species within the palm family to synthesise fats of similar composition is shown very strikingly by a comparison of the fatty acid mixtures of the five new fats examined in this paper, (i) with each other, and (ii) with the recent analyses already mentioned of the component acids from coconut, palm kernel, cohune nut, murumuru and *Attalea funifera* kernel-fats.

Table I.

	% acids.							
	Cap- rylic	Capric	Lauric	My- ristic	Pal- mitic	Stearic	Oleic	Lin- oleic
<i>Acrocomia sclerocarpa</i>	7.8	5.6	44.9	13.4	7.6	2.6	16.5	1.6
<i>Manicaria saccifera</i>	5.3	6.6	47.5	18.9	8.2	2.4	9.7	1.4
<i>Astrocaryum Tucuma</i>	1.3	4.4	48.9	21.6	6.4	1.7	13.2	2.5
<i>Astrocaryum Murumuru</i> [Saraiva, 1929]	1.1	1.6	42.5	36.9	4.6	2.1	10.8	0.4
<i>Maximiliana caribaea</i>	(?)	5.0	47.0	21.0	9.0	(?)	(18)	
<i>Attalea excelsa</i>	(Similar composition to that of <i>Acrocomia sclerocarpa</i>)							
Cohune (<i>Attalea cohune</i>)	7.5	6.6	46.4	16.1	9.3	3.3	9.9	0.9
[Hilditch and Vidyarthi, 1928]								
<i>Attalea funifera</i> [Heiduschka and Agsten, 1930]	6.5	2.7	45.9	19.9	6.9	—	18.1	—
Coconut I [Collin and Hilditch, 1928]	7.9	7.2	48.0	17.5	9.0	2.1	5.7	2.6
Coconut II	7.8	7.6	44.9	18.1	9.5	2.4	8.2	1.5
Palm kernel [Collin and Hilditch, 1928]	2.7	7.0	46.9	14.1	8.8	1.3	18.5	0.7

In each case the same range of fatty acids is present, but each genus, as it were, asserts its individuality by slight variations in particular components while conforming to the same general type. The comparative constancy of the major constituent of the mixtures, lauric acid, is particularly noticeable and, similarly, capric, myristic and palmitic acids all occur within fairly narrow ranges. The apparent absence of caprylic and stearic acids from the fat of *Maximiliana caribaea* may well be attributed to incomplete separation of the mixture due to lack of material.

GLYCERIDE STRUCTURE OF THE KERNEL-FATS OF *ACROCOMIA SCLEROCARPA* MART., *MANICARIA SACCIFERA* GAERTN. AND *ASTROCARYUM TUCUMA* MART.

The determination of the content of fully-saturated glycerides in these three fats and the consequent disclosure of the extent to which the unsaturated acids are distributed throughout the glyceride molecules has demonstrated that the usual tendency towards even distribution, characteristic of kernel-fats, holds throughout this group. The numerical expression of the extent of dispersion of the unsaturated acids, the "association ratio" [Collin and Hilditch, 1929], is in fair agreement in all three cases with the corresponding values for coconut and palm kernel-fats and indicates that all these fats possess similar glyceride structures. The case for the absence of triolein from coconut and palm kernel-fats has been put in a previous paper [Collin and Hilditch, 1928] and applies

with equal force to the present fats. Assuming triolein to be absent, the glyceride composition of the fats may be expressed in terms of saturated glycerides, mono-unsaturated disaturated glycerides, and di-unsaturated monosaturated glycerides (the figures refer to mols. per 100 mols. of fat) (Table II).

Table II.

	Fully-saturated glycerides	Mono-unsaturated disaturated	Di-unsaturated monosaturated
<i>Acrocomia sclerocarpa</i>	69	21	10
<i>Manicaria saccifera</i>	82	11	7
<i>Astrocaryum Tucuma</i>	73	18	9

Acrocomia sclerocarpa (*Gru-gru*) kernel-fat.

This tree, known locally as the gru-gru palm, is indigenous to Central America and Brazil, although the specimen from which this sample of nuts was taken grew in Trinidad. The nuts were kindly supplied by the Department of Agriculture, Port of Spain, Trinidad, to whom we are also indebted for the specimens of *Manicaria saccifera* and *Maximiliana caribaea* nuts.

The nuts contained 26 % of kernel, which, by extraction with low-boiling light petroleum, yielded 44.4 % of a white soft fat having saponification equivalent 222.3, acid value 0.6, iodine value 17.1, unsaponifiable matter 0.45 % and M.P. (open tube) 24°. The corresponding mixed fatty acids, containing the unsaponifiable matter, had saponification equivalent 210.4, iodine value 18.0, and setting-point 22°. A quantitative analysis of the component fatty acids has not been published but various workers have determined the constants of the fat, and obtained values for the saponification equivalent varying between 220–230, iodine value 16–21, M.P. 21–32.5° and Reichert-Meissl value 5.7–7.2.

The component fatty acids, determined by the ester fractionation process as described [Collin and Hilditch, 1928], are given in Table III.

Table III.

	% acids	
	Weight	Mols.
Caprylic	7.8	11.5
Capric	5.6	6.9
Lauric	44.9	47.6
Myristic	13.4	12.2
Palmitic	7.6	6.3
Stearic	2.6	1.8
Oleic	16.5	12.5
Linoleic	1.6	1.2

Fully-saturated glycerides. The fat (99.6 g.) was dissolved in acetone and treated with potassium permanganate in the usual manner. On working up the oxidation product there were obtained 69.4 g. crude fully-saturated glycerides (iodine value 0.3). After further purification with boiling aqueous potassium carbonate, the crude product was separated into (a) 42.9 g. of saturated glycerides (saponification equivalent 208.3, acid value 1.4), (b) 20.6 g. of saturated glycerides (acid value 2.2), and (c) 3.84 g. of acid product. This corresponds with a content of fully-saturated glyceride in the original fat of 64.5 % by weight or of 69.1 mols. per 100 mols. of fat. It also indicates an "association ratio" in the mixed saturated-unsaturated glycerides of 1.29 mols. of saturated acid with 1 mol. of unsaturated acid.

Manicaria saccifera kernel-fat.

As in the case of *Acrocomia sclerocarpa*, the palms from which the nuts were obtained, although indigenous to Central America and Brazil, were grown in Trinidad.

The kernels formed 15 % of the nut and gave 57.7 % of a soft white fat on extraction with low-boiling light petroleum. The fat possessed the following analytical characteristics: saponification equivalent 221.8, acid value 0.6, iodine value 10.7, unsaponifiable matter 0.05 %, and M.P. (open tube) 27.1°. The mixed fatty acids, containing the unsaponifiable matter, had saponification equivalent 208.0, iodine value 11.4 and setting-point 24.3°. Again there is no quantitative analysis of the fatty acids on record, but there is a reference in the literature [Olien en Vetten, 1919] to a specimen of this fat with a saponification equivalent of 232.2, iodine value 19 and M.P. 30°.

The results of the detailed analysis of the mixed fatty acids from the present specimen are given in Table IV.

Table IV.

	% acids	
	Weight	Mols.
Caproic	Trace	—
Caprylic	5.3	7.8
Capric	6.6	8.0
Lauric	47.5	49.9
Myristic	18.9	17.4
Palmitic	8.2	6.7
Stearic	2.4	1.8
Oleic	9.7	7.3
Linoleic	1.4	1.1

Fully-saturated glycerides. Permanganate-acetone oxidation of the fat (25.9 g.) yielded 20.6 g. of crude fully-saturated material; this was resolved, by further purification, into (a) 19.50 g. saturated glycerides (saponification equivalent 214.5, acid value 0.5), (b) 0.71 g. saturated glycerides (acid value 2.9) and (c) 0.39 g. acid product. The content of fully-saturated glyceride is therefore 79.5 % (weight) or 82.3 % (mols.), corresponding to an association ratio of 1.16 to 1.

Astrocaryum Tucuma kernel-fat.

The natural habitats of this palm are Brazil, Central America and the West Indies, but the palms from which the fat now examined was obtained were grown in Malaya. The nuts were collected through the kindness of Messrs E. Boustead and Co. (who also supplied the nuts of *Attalea excelsa*) from the Malakoff Rubber Estates.

The decorticated kernels yielded 39.8 % of a white fat, rather harder than in the previous instances, of saponification equivalent 230.3, acid value 1.8, iodine value 15.8, unsaponifiable matter 0.4 % and M.P. (open tube) 30.3°. The mixed fatty acids had saponification equivalent 218.9, iodine value 16.6 and setting-point 26.8°. The values recorded in the literature vary between the following limits: saponification equivalent 225–226, iodine value 9.4–11.6, M.P. 30.5–35.5°.

The results of the fractionation of the esters of the mixed fatty acids in the usual manner are given in Table V.

Fully-saturated glycerides. Oxidation of the fat (98.6 g.) with potassium permanganate in acetone yielded 72.1 g. of crude saturated glycerides which

Table V.

	% acids	
	Weight	Mols.
Caprylic	1.3	1.9
Capric	4.4	5.6
Lauric	48.9	53.1
Myristic	21.6	20.6
Palmitic	6.4	5.5
Stearic	1.7	1.3
Oleic	13.2	10.1
Linoleic	2.5	1.9

were further separated into (a) 61.7 g. saturated glycerides (saponification equivalent 220.5, acid value 0.6), (b) 4.40 g. saturated glycerides (acid value 2.6) and (c) 2.24 g. acid product. The original fat thus contained 69.7 % (weight) or 72.8 % (mols.) of fully-saturated glycerides and its "association ratio" was 1.25 : 1.

Maximiliana caribaea kernel-fat.

This species is also indigenous to Central America and Brazil, but the nuts studied were grown in Trinidad. The nuts, which were obviously immature, contained 59 % of kernel of which only 4.8 % was fat. The fat had the following characteristics: saponification equivalent 236.8, acid value 20.2, iodine value 22.7, unsaponifiable matter 0.23 %. There are no previous references to it in the literature. As already explained, the amount of fat at disposal only permitted a rudimentary fractionation of the esters of the mixed fatty acids to be undertaken. The results suggested the following approximate proportions of component fatty acids (Table VI).

Table VI.

	% acids	
	Weight	Mols.
Capric	5	6
Lauric	47	50
Myristic	21	21
Palmitic	9	8
Stearic	?	?
Oleic	18	15
Linoleic		

Attalea excelsa (Pallia palm) kernel-fat.

This batch of nuts came from palms grown in Malaya, but the tree is a native of Brazil. The kernel formed only 3 % of the nut and yielded 62.6 % of fat with saponification equivalent 231.7, iodine value 18.2, M.P. (open tube) 25.5°. The mixed acids had saponification equivalent 218.6, iodine value 19.3. Previous workers [Wittka, 1925; Allan and Moore, 1925] give the following values: saponification equivalent 218-241, iodine value 8-16, M.P. 18-19°.

The primary fractionation of the mixed esters may be compared with the primary fractionation of the mixed esters of *Acrocomia sclerocarpa* (Table VII).

The similarity in range of boiling-point, weights and analytical constants between the two sets of fractions is sufficient indication that the two mixtures are broadly the same in composition. The fat from *Attalea excelsa*, however, is not so rich in the group of acids below C₁₂ as *Acrocomia sclerocarpa* fat, and this is balanced by its increased content of myristic, palmitic and stearic acids.

Table VII.

<i>Attalea excelsa</i> Mixed esters					<i>Acrocomia sclerocarpa</i> Mixed esters				
	B.P.	Weight %	S.E.	I.V.		B.P.	Weight %	S.E.	I.V.
1	55-88°	24	196.6	0.5		50-95°	29	188.6	0.3
2	88-90	25	217.6	0.7		95-105	30	217.7	0.5
3	90-130	31	252.8	17.2		105-130	18	245.6	15.8
4	Residue	20	295.9	60.3		Residue	23	291.7	64.2

Coconut oil.

The coconut oil used was taken from a refined commercial specimen supplied by Messrs Lever Brothers, Ltd., and had saponification equivalent 217.2, iodine value 9.7 and acid value 0.3. The experimental determination of the fatty acid mixture and the fully-saturated glycerides was carried out by Dr R. Child; the final composition of the component acids is given in Table VIII.

Table VIII.

	% acids	
	Weight	Mols.
Caproic	Trace	—
Caprylic	7.8	11.2
Capric	7.6	9.2
Lauric	44.9	46.5
Myristic	18.1	16.5
Palmitic	9.5	7.7
Stearic	2.4	1.8
Oleic	8.2	6.0
Linoleic	1.5	1.1

Fully-saturated glycerides. Permanganate-acetone oxidation of the fat (400 g.), yielded (a) 244 g. saturated glycerides (saponification equivalent 211, acid value nil), (b) 82 g. saturated glycerides (acid value 6.6), and (c) 57.6 g. acid product. The original fat therefore contained 80.7 % (weight) or 84 % (mols.) of fully-saturated glycerides. The corresponding "association ratio" of saturated to unsaturated acids in the mixed non-fully-saturated glycerides is 1.4 : 1.

This result is of interest when compared with those for the only other coconut-fat glycerides previously examined [Collin and Hilditch, 1928]. The specimen of fat then studied contained slightly more combined oleic and linoleic acids than the present one and, correspondingly, slightly less fully-saturated glycerides. The ratio of saturated to unsaturated acids in the non-fully-saturated glycerides was, however, the same in both cases. In seed-fats wherein unsaturated acids do not form more than about 40 % of the total fatty acids, the amount of fully-saturated glycerides is determined by the excess of saturated acids over and above the quantity necessary to link with unsaturated acids in mixed glycerides with an "association ratio" of about 1.3 to 1.4 : 1.

Cordial thanks are offered to Mr C. D. V. Georgi and to Messrs E. Boustead and Co. for assistance in obtaining the specimens from Malaya; to Mr J. Williams and the Department of Agriculture, Trinidad, for help in the collection of the Trinidad nuts; to Messrs Lever Brothers, Ltd.; and to Prof. J. McLean Thompson, who kindly identified the "pallia palm" nuts as those of *Attalea excelsa* Mart.

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CLXXXV. FATTY ACIDS FROM THE LARVA-FAT OF THE BEETLE *PACHYMERUS DACTRIS* L.

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(Received July 12th, 1933.)

WHEN the nuts of *Manicaria saccifera* Gaertn. [Collin, 1933] were being prepared for extraction, many of the kernels were found to contain living grubs, which were identified as the larvae of the beetle *Pachymerus dactris* Linn. This insect has also been found in the kernels of the cohune nut (*Attalea cohune*) and is a common pest of these species [Hilditch and Vidyarthi, 1928]. The larvae had eaten all the fat-bearing endosperm, and in some cases had undergone metamorphosis into the beetles, which were boring their way through the hard shell of the nut. The fat from the larvae was extracted and obtained in sufficient quantity for a rudimentary analysis by the ester-fractionation method.

8.5 g. of semi-solid fat were obtained from 53 larvae (18 g.) by soaking them in acetone, drying, extracting with low-boiling petroleum and combining the acetone and petroleum extracts. This had saponification equivalent 260.7, iodine value 37.4 and acid number 3.2, and was analysed in the usual way by distilling the methyl esters of the "liquid" and "solid" acids (obtained by lead salt separation) from a 50 cc. flask with column into small receivers carried on a rotating support inside a vacuum desiccator. The quantities dealt with were so small that the accuracy of the analysis is not of a very high order.

The acids were separated into "solid" acids (57.5 %, iodine value 3.0) and "liquid" acids (42.5 %, iodine value 70.9): the calculated approximate composition of the mixed esters is given below:

								Esters % by weight
Lauric	24
Myristic	21
Palmitic	8
Oleic	32
Linoleic	3
Stearic, oleic or linoleic (present in residual fractions)								12

Comparing these figures with those for the kernel fat of *Manicaria saccifera* [Collin, 1933], it appears that the acids of lower molecular weight (of which lauric acid is the chief) are present in the larva-fat in only about half the amount in which they occur in the kernel-fat, while oleic and linoleic acids probably form about 40 % of the mixed acids in the larva-fat, as compared with only 11 % in the kernel-fat. This rather suggests that the insect has derived its fat partly by direct assimilation of the preformed vegetable fat, and partly by synthesis from carbohydrate (or other non-fatty) components of the kernel. If this be the case, it would appear that the development of fat in insects may follow a course not very different from that which takes place in the larger land vertebrates, such as the pig.

Unfortunately there is little information in the literature on insect fats. The cocoon of the silkworm, *Bombyx mori* Linn., contains about 25 % of fat

consisting of mixed glycerides of palmitic, oleic, linoleic and linolenic acids, and the mixed fatty acids include about 25 % of saturated (palmitic and stearic) with about 22 % oleic, 38 % linoleic and 15 % linolenic acids [Tsujimoto, 1908; 1916; Suzuki and Yokoyama, 1928]. The only other instances of larva-fats are those from two insects of unknown origin [Desvergues, 1920], the mixed acids of which had a mean molecular weight of 263-269 and contained unsaturated acids (50-60 %) with iodine values of 140 and 95. The general characteristics of a few fats of insects belonging to the Coleoptera, Diptera and Orthoptera have been reported, from which it appears that the major components are oleic and linoleic acids and that, although palmitic acid is probably also present, acids of lower molecular weight are absent. An exception to this statement is, however, found in the fat of *Pemphigus* species (Aphidae), the acids of which are reported by Schultz [1922] to have a mean molecular weight of 218 and to include butyric, caprylic and lauric as well as palmitic acids.

On the whole, it seems likely that insects, in the larval as well as mature state, lay down fats somewhat similar in type to those produced by mammals, and that, like the latter, they can assimilate fats present in their diet and also synthesise fat from other constituents of the food. More complete study of insect fats than has hitherto been made might well be of interest from a biochemical standpoint.

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CLXXXVI. THE COMPONENT FATTY ACIDS OF RAT BODY FATS.

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(Received August 19th, 1933.)

DETAILED quantitative information upon the composition of the fatty acids combined in the depot fats of animals is abundant in the cases of the ox, sheep and pig, but is otherwise still scanty, except in a few isolated instances, such as the loin fat of the reindeer [Baughman *et al.*, 1929] and the body fat of the horse [Heiduschka and Steinruck, 1921; Grossfeld, 1931]. These two fats appear to fall into the same category as the tallows and lards, in that their component acids contain about 30 % (weight) of palmitic acid and substantial, but variable, proportions of stearic acid, the amount of the latter varying more or less inversely with that of the oleic acid present [*cf.* Banks and Hilditch, 1932]. Of bird depot fats, those of the goose [Bömer and Merten, 1922; Grossfeld, 1931] and the hen [Grossfeld, 1931] have been examined by resolution into "solid" and "liquid" acids by lead salt separation (Twitchell), or by oxidation (Bertram). The bird fats seem to belong to a different type from the animal fats mentioned, since the analyses quoted indicate the presence of only small amounts of stearic acid (usually not more than 5 %), and the proportion of palmitic acid is also lower (20–25 %). These differences, which are confirmed and extended, in the case of hen fats, by studies by the ester-fractionation method now proceeding in this laboratory, point to the desirability of collecting detailed quantitative data for the depot fats from a much wider variety of animals than that dealt with up to the present.

As a contribution to this end, we are able to communicate the results of analyses, by the ester-fractionation process, of four rat body fats and of the perinephric fat from wild rabbits (the latter analysis having been carried out by Mr J. R. Vickery in 1928, but not hitherto published). Burr and Burr [1930] pointed out that rats appear not to lay down linoleic acid in their reserve fats and suggested that they are unable to synthesise linoleic acid; according to these authors, the absence of fats containing linoleic acid from the diet causes the animals to develop the condition known as "scaly tail." Gregory and Drummond [1932] studied the depot and liver fats of rats fed on various diets by examining the analytical characteristics of the mixed "liquid" or mainly unsaturated acids obtained from the Twitchell lead salt separation. They confirmed Burr and Burr's observations that, with fat-free diets, the body fats were substantially devoid of linoleic acid, but found that this acid was present in the liver fats, from which it would follow that rats are able to synthesise this acid but do not deposit it in the reserve fat.

This peculiarity, the extensive use made of rats in nutritional experiments, and the desire already mentioned to extend the range of information on animal depot fat component acids, made us welcome an offer by Prof. Drummond to

place at our disposal, for study by the ester-fractionation method, specimens of depot fats collected from rats which had been fed (a) on mainly fat-free diets and (b) on diets supplemented by cod-liver oil. We are further indebted to Prof. Drummond for the information which follows regarding the origin of these four rat fats.

Each sample represented the whole of the adipose tissue fat dissected out from the subcutaneous, perinephric and mesenteric areas of a number of animals. Each rat yielded in all 5–6 g. of fat, and the total amount of available material in each specimen varied from 40 to 70 g. The latter quantity would have been sufficient for an accurate determination of a simple mixture of palmitic, stearic, oleic and linoleic acids; it will be seen, however, that the analyses were complicated by the apparent presence of small proportions of an unsaturated fatty acid of lower molecular weight than oleic acid (probably palmitoleic acid). Owing to this, it would be necessary to work with much larger quantities of material than were available in order to obtain data of the desired order of accuracy. The figures given for palmitic, stearic and linoleic acids, however, are probably very close to the truth, whilst that for oleic acid must be regarded as uncertain to within perhaps 3 or 4 units %. Fortunately, the general and characteristic features of the component acids are quite evident in spite of the minor uncertainties thus unavoidably present in some of the numerical data.

Three samples of rat fats were originally supplied.

Fat A was derived from rats fed on "fat-free" diets, but most of the animals had received daily doses of about 15 mg. of ethyl laurate as a carrier of vitamin A or carotene over periods varying from 3 to 7 weeks.

Fat B was from rats fed on different diets, none of which contained unsaturated fats. Most of these animals were on very low fat diets, but in some cases 10 % of the diet consisted of hydrogenated cottonseed oil; nearly all had supplements conveyed in ethyl laurate (as in A).

Fat C was from rats which had received diets containing from 2 to 15 % (in most cases 5 %) of cod-liver oil over periods of 10 weeks.

In view of the small amount of C available (*ca.* 40 g.), and of the interesting results disclosed, Prof. Drummond kindly undertook a special experiment in which 12 rats were fed for 12 weeks on a ration of "Glaxo casein" 20 %, rice starch 73 %, salt mixture 5 % and cod-liver oil 2 %. The resulting fat (D, 70 g.), however, did not reproduce the characteristics of fat C, possibly owing to the low proportion of cod-liver oil used.

Each fat was saponified, and the mixed fatty acids were divided into "solid" (mainly saturated) and "liquid" (mainly unsaturated) acids by the usual lead salt separation from alcohol (in presence of 0.5 % acetic acid). Each group of acids was then converted into methyl esters and fractionated under low pressure. The fractionation data are quoted in full (Table III) in order to make clear the basis of the deductions as to the composition of the mixed acids.

The general characteristics of the fats, all of which were almost entirely colourless and not completely solid at room temperature, may first be recorded (Table I).

Table I.

Fat	Diet	Saponification equivalent	Iodine value	Acid value	Setting-point of mixed fatty acids
A	"Fat-free"				
B	Mainly "fat-free"	281.0	60.5	34.8	37.2°
C	2–15 % cod-liver oil	283.3	57.3	17.0	38.2
D	2 % cod-liver oil	284.2	74.6	18.9	36.2
		280.3	57.4	20.0	39.6

The proportions of "solid" and "liquid" acids obtained from the mixed fatty acids of each fat in the preliminary lead-salt separation are given in Table II.

Table II.

Fat	"Solid" acids		"Liquid" acids	
	g.	%	g.	%
A	14.3	30.7	32.3	69.3
B	18.9	33.9	36.8	66.1
C	9.7	28.4	24.5	71.6
D	21.2	35.3	38.8	64.7

The details of the ester-fractionations are given in Table III. The last fraction in each case represents the residue left in the distillation bulb. In the distillations of the "liquid" esters the residues, after saponification, are freed from unsaponifiable matter, and the equivalents and iodine values of the recovered acids (free from unsaponifiable) are re-determined in order to estimate

Table III. *Fractional distillation of methyl esters of the "solid" and "liquid" acids.*

No.	"Solids"			"Liquids"		
	g.	Sap. Eq.	I.V.	g.	Sap. Eq.	I.V.
From fat A ("fat-free" diets).						
1	3.04	264.4	3.2	5.67	268.9	75.9
2	4.11	269.8	4.0	4.49	279.2	85.1
3	3.54	273.0	5.9	4.90	285.7	86.8
4	2.97	282.0	12.0	5.52	290.2	87.8
5	—	—	—	4.77	294.0	88.5
6	—	—	—	3.40	306.3	88.7
	13.66	—	—	28.75	(296.6)	(88.6) (Unsap.-free)
From fat B (mainly "fat-free" diets).						
1	3.33	264.8	2.0	4.41	273.0	75.5
2	3.15	268.5	2.5	4.55	282.4	82.1
3	2.79	269.1	3.4	4.38	286.7	84.2
4	3.86	271.8	4.2	5.39	292.3	84.3
5	2.64	281.5	11.3	4.82	294.1	85.6
6	—	—	—	5.02	296.3	85.8
7	—	—	—	4.04	305.0	89.3
	15.77	—	—	32.61	(296.7)	(88.0) (Unsap.-free)
From fat C (2-15 % cod-liver oil in diets).						
1	2.60	266.9	1.7	3.67	276.5	77.6
2	1.84	269.1	3.4	3.80	286.3	88.4
3	2.35	270.9	4.7	3.90	290.3	88.2
4	2.74	281.0	11.3	3.84	296.0	95.7
5	—	—	—	5.06	300.5	104.6
6	—	—	—	3.95	320.4	155.8
	9.53	—	—	24.22	(314.0)	(150.8) (Unsap.-free)
From fat D (2 % cod-liver oil in diets).						
1	2.94	266.2	0.9	12.12	277.4	80.8
2	3.70	267.7	1.9	4.62	288.0	87.1
3	3.60	269.6	2.1	4.52	289.6	87.4
4	4.83	271.9	3.6	4.18	295.6	88.1
5	4.31	280.3	9.9	4.66	294.7	88.8
6	—	—	—	6.12	303.8	93.9
	19.38	—	—	36.22	(295.5)	(91.4) (Unsap.-free)

the amount of unsaponifiable material present. The values so obtained (stated in terms of the corresponding methyl esters) are added in brackets in Table III. (All the unsaponifiable matter passed, in solution in alcohol, into the "liquid" acids.)

When the composition of the lower-boiling fractions of the "liquid" methyl esters from fats A, B and C was estimated on the assumption that no unsaturated esters were present of lower molecular weight than the C_{18} ester-mixtures (of respective iodine values 88.5, 85.8 and 95.7), it was found that the calculated mean equivalents of the saturated material in these fractions were much lower than that of methyl myristate. The lowest-boiling fraction of "liquid" esters from fats which have undergone oxidative rancidity often exhibits this feature, although not to the same extent as in the present instances; moreover, the rats now described had not become oxidised appreciably. An alternative explanation lay in the presence of an unsaturated acid of lower molecular weight than oleic acid and, although the amount of material at our disposal was insufficient to permit of any positive identification of such acid, some indirect evidence was obtained by modifying the fractional distillation of the "liquid" esters from fat D.

In this instance, a considerably larger first (lowest-boiling) fraction than usual was collected (12.12 g., or about one-third of the whole, and containing nearly all the esters of lower molecular weight than methyl oleate-linoleate). The amount (8.7 g.) not required for direct analysis was dissolved in acetone and oxidised with potassium permanganate in order to convert all unsaturated esters into acidic products; the residual neutral product was then re-submitted to the same oxidation process. After the double oxidation, the remaining neutral ester (0.97 g.) still possessed a small iodine value (5.1) and the fatty acids combined therein (after boiling with water to remove traces of nonoic, azelaic, *etc.*, acids) were solid and had a mean molecular weight somewhat higher than that of myristic acid. After correcting for the small residual iodine value of the product obtained from the oxidations, the corresponding weight of saturated ester present in the original fraction (12.12 g.) was 1.3 g. Assuming this to be methyl myristate (although actually small proportions of methyl palmitate were probably also present), the mean molecular weight of the unsaturated esters also present was about 282; calculated in terms of a mixture of methyl palmitoleate and oleate-linoleate (of mean iodine value 88.8), this corresponds with the possible presence of 4.8 g. methyl palmitoleate and 6.0 g. methyl oleate-linoleate in this fraction.

On this basis, the component fatty acids of fat D were made up approximately of myristic (4 %), palmitic (30 %), stearic (2.5 %), palmitoleic (?) (8.5 %), oleic (53 %) and linoleic (2 %) acids.

Since, therefore, the presence of small amounts of an unsaturated acid of lower molecular weight than oleic acid was definitely indicated, we have calculated the component fatty acids of all four fats on the basis that the "liquid" ester fractions are made up of methyl myristate, palmitoleate and oleate (or oleate-linoleate). The results are given in Table IV.

We are aware that the impossibility of definitely identifying an unsaturated acid of lower molecular weight than oleic acid in the present series renders the foregoing calculation somewhat unsatisfactory, and we therefore add, for fats A, B and C (Table V), the results of the alternative calculation, in which the absence of unsaturated acids with less than 18 carbon atoms in the molecule is assumed. This, however, involves the presence of small amounts of saturated acids (lauric and even capric) of lower molecular weight than myristic acid;

and we have not been able to detect the presence of any acids of lower molecular weight than myristic acid.

Table IV. *Component fatty acids of rat body fats A, B, C, D.*
(Assuming the presence of palmitoleic acid.)

Diet ...	A "Fat-free" %	B Mainly "fat-free" %	C 2-15 % cod-liver oil %	D 2 % cod-liver oil %
Myristic acid	5	4.5	5	4
Palmitic acid	24	28	23	30
Stearic acid	3	2	2.5	2.5
Palmitoleic (?) acid	8	7	5.5	8.5
Oleic acid	58	58.5	51.5	53
Linoleic acid	2	—	4	2
C ₂₀₋₂₂ unsaturated acids*	—	—	8.5	—

* In fat C the fractionation data clearly indicated the presence of highly-unsaturated acids of the C₂₀ and C₂₂ series in the highest-boiling and residual fractions of the "liquid" esters; their amount was approximately calculated by the same means as that employed in similar cases which have been encountered in pig body fat analyses [Banks and Hilditch, 1932].

Table V. *Component fatty acids of rat body fats A, B, C.*
(Assuming absence of any unsaturated acids of lower carbon content than C₁₈.)

Diet ...	A "Fat-free" %	B Mainly "fat-free" %	C 2-15 % cod-liver oil %
Myristic and lower saturated acids	4.5	4	5
Palmitic acid	24	28	23
Stearic acid	3	2	2.5
Oleic acid	66.5	66	54.5
Linoleic acid	2	—	6.5
C ₂₀₋₂₂ unsaturated acids	—	—	8.5

DISCUSSION.

It will be understood, from what has already been said, that the values given for the amounts of oleic acid present depend upon whether a palmitoleic acid, or even some unsaturated acid of still lower molecular weight, is also present; the figures for oleic acid in Tables IV and V together represent, however, the extreme possible limits. Apart from this uncertainty, a number of points of interest emerge from the analyses.

Body fats (A and B) of rats fed on mainly fat-free diets. The results amply confirm the observations of the previous investigators, namely, that rats receiving no linoleic acid in their fatty diet deposit reserve glycerides from which that acid is almost or completely absent.

A feature which interests us especially, however, is the relative amounts of palmitic acid and of stearic acid present in these fats as compared with the corresponding proportions in the body fats of pigs, oxen and sheep. The proportion of palmitic acid is, indeed, of much the same order in all cases: expressed in molar percentages, the united amounts of palmitic and myristic acids (the latter being 4-5 % in each case) in the four rat fats lie between 30 and 35 %, a figure which is almost identical with that observed throughout a large series of lards and tallowes [cf. Banks and Hilditch, 1931; 1932]. The proportions of stearic acid are, on the contrary, strikingly different in the body fats of the rat and of pigs, sheep and oxen. In the latter group stearic is definitely a major

component acid, occurring in widely varying amounts. In the rat body fats it is equally clearly a minor component and occurs in proportions relatively small compared with those of palmitic or oleic acid.

In other words, the component acids of the rat body fats consist essentially of 25-30 % of palmitic acid, the remainder (apart from small proportions (4-5 %) of myristic and 2-3 % of stearic acid) being made up entirely of unsaturated acids, in which oleic acid predominates, and from which linoleic acid is practically absent. In consequence, about 65-70 % of the rat fat component acids belong to the unsaturated (oleic) series, whereas in lards and tallow the unsaturated acids vary from less than 40 %, and rarely exceed 55 %, of the mixed fatty acids.

Fully-saturated glycerides were only present in very small proportions in either fat, specimen A containing about 2.5 % and specimen B about 3.5 %. The fatty acids present in the fully-saturated components had mean molecular weights of 260 (A) and 258 (B) (palmitic acid, mol. wt., 256), so that these consisted almost entirely of tripalmitin. This is, of course, another difference from the depot fats of pigs, sheep and oxen, since the latter, in addition to containing greater amounts of stearic acid, also contain fully-saturated components in proportions increasing with those of the stearic acid present, and consisting for the most part of mixed palmitostearins [Banks and Hilditch, 1931; 1932].

Body fats (C and D) of rats fed on diets which included cod-liver oil. The specimen C, which was derived from rats whose diet had contained from 2 to 15 % of cod-liver oil, was differentiated from the rest by the presence of a certain amount (about 8 %) of the highly-unsaturated acids of the C_{20} and C_{22} series which are present in cod-liver oil. Apart from this, the remaining acids appeared to be present in much the same relative proportions as when the diet of the animals was mainly fat-free, but the presence of linoleic acid had become definitely noticeable (4 %). Nevertheless, the linoleic acid content is surprisingly small when compared with that of the deposited unsaturated C_{20-22} acids, since each type of acid is present in much the same proportions in cod-liver oil [Guha *et al.*, 1930].

This feature is also peculiar in view of the observation of Gregory and Drummond [1932] that the body fat of rats fed on diets including olive oil contained unsaturated acids of which linoleic acid formed from 7 to 10 % (*i.e.* practically the same proportion as in olive oil itself). With the olive oil diets used by these workers, there was, moreover, a considerable decline in the saturated acid content of the body fats, namely, from 32 % (fat-free diet) to 22 % (12 % olive oil in diet) and 11 % (50 % olive oil in diet). The fact that, in fat C, the total content of saturated acids (30.5 %) was only slightly less than that in the fats from animals on mainly "fat-free" diets (32-35 %), suggests either that the added cod-liver oil was not freely utilised, or, more probably, that the average amount of cod-liver oil in the diets (about 5 %, *cf.* p. 1376) was insufficient to produce any marked alteration in the body fat. In the case of the pig, for example, addition of cottonseed oil to the diet does not cause definite modification of the body fat until the oil forms more than 4 % of the diet [Ellis *et al.*, 1931].

The body fat D (from rats which had received a diet including only 2 % of cod-liver oil) was of practically the same composition as fats A and B, and showed no increase in linoleic acid and no perceptible amount of the higher unsaturated acids. The only indication of the presence of the latter was a slightly increased iodine value (93.9) in the residual fraction of "liquid" esters

(Table III), but this was not accompanied by any perceptible increase in the equivalent (295.5) of these esters (after correcting for unsaponifiable matter). In this experiment, therefore, the characteristic cod-liver oil acids did not appear in the depot fat.

The perinephric fat of the (wild) rabbit. We are able to add some details as to the adipose tissue fat of another rodent, from an ester-fractionation analysis (made in this laboratory some years ago by Mr J. R. Vickery) of the component fatty acids of the perinephric fat from wild rabbits. This was a yellow-coloured, very unsaturated fat, almost liquid at the ordinary temperature, with a saponification equivalent of 285.2 and an iodine value of 124.0. The component acids were made up of myristic (4.5 %), palmitic (23 %) and stearic (4 %) acids, the remainder (68.5 %) consisting of unsaturated acids (apparently almost wholly of the C_{18} series) with a mean iodine value of 189.3. The latter acids yielded solid hexabromo-addition products equivalent to a content of 9 % of linolenic acid in the total fatty acids; it is therefore quite possible that the total amount of linolenic acid in the mixed acids approached 15–20 %, since the yield of "insoluble hexabromides" usually corresponds with not more than about 50 % of the linolenic acid present.

This rabbit perinephric fat closely resembles the rat fats in its proportion of palmitic, stearic and myristic acids and in its general content of about 30 % saturated and 70 % unsaturated acids. It is also similar in its fully-saturated glyceride content, namely, about 6–7 %, almost wholly tripalmitin (the latter triglyceride was also obtained from rabbit fat by fractional crystallisation by Klimont [1912]). The nature of the unsaturated acids in rat fats and in rabbit fats is, however, wholly dissimilar: whereas, in that of the rat, linoleic acid is practically absent, the rabbit fat described contained large amounts of this component (35–50 % of the mixed acids) and also appreciable quantities of linolenic acid.

SUMMARY.

The body fat of the rat belongs to a group in which the component acids are made up of palmitic (25–30 %), myristic (4–5 %) and small amounts of stearic (2–3 %), the remainder (about 65–70 %) consisting chiefly of oleic acid, apparently with small quantities of a palmitoleic acid; linoleic acid is, however, almost entirely absent.

When the diet of the animals includes cod-liver oil, some of the highly-unsaturated acids of the C_{20} and C_{22} series may occur in the depot fats, but otherwise little modification occurs, and the proportion of linoleic acid is only slightly increased.

The perinephric fat of a wild rabbit contained similar amounts of myristic (4.5 %), palmitic (23 %) and stearic (4 %) acids, but the unsaturated acids (68.5 %) differed widely from those of the rat fats, linoleic acid being the main component, while considerable amounts of linolenic acid were also present.

Fully-saturated components (almost wholly tripalmitin) were present only in small proportions in the body fats of either the rat (2–3 %) or the rabbit (6–7 %).

There seem to be at least two well-defined groups of depot fats characteristic of land (*i.e.* non-aquatic) animals.

(a) Fats with component fatty acids containing about 30–35 % saturated (usually 25–30 % palmitic) acids and for the rest unsaturated acids of varying type (oleic, linoleic, *etc.*). In these stearic acid is a minor component (not exceeding about 5 %), and fully-saturated components are only present in small

proportions and consist mainly of tripalmitin. The rodent body fats discussed in this paper, and probably also bird depot fats, fall in this category.

(b) Fats with component fatty acids made up of about 25–30 % palmitic and small amounts of myristic acids, the remaining 65–70 % consisting of stearic, oleic and linoleic acids. In this group (to which belong the depot fats of the pig, ox, sheep, horse, reindeer) the proportion of stearic acid is variable but usually important. The greater the amount of stearic acid (with correspondingly less oleic acid), the greater is the proportion of fully-saturated glycerides in the fat; in this group, these consist for the most part of mixed palmitostearins.

The persistence with which the palmitic acid content remains in the neighbourhood of 25–30 %, and that of myristic acid at about 4–5 %, throughout both groups is noteworthy.

We desire to thank the Department of Scientific and Industrial Research for a grant in aid of this work.

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CLXXXVII. THE OXYTOCIC HORMONE OF THE
POSTERIOR LOBE OF THE PITUITARY GLAND.
IV. THE ACTION OF PREPARATIONS OF ANIMAL PRO-
TEOLYTIC ENZYMES, AND SOME OBSERVATIONS ON
THE NATURE OF THE HORMONE.

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(Received July 12th, 1933.)

IN Part III of this series Gulland and Macrae [1933] described the action of preparations of some plant proteolytic enzymes on the oxytocic hormone. They showed that preparations of yeast dipeptidase, aminopolypeptidase and proteinase, and of papain, contained an inactivating enzyme which had its p_H optimum at 7.4. This enzyme, which was not identified, reduced the oxytocic activity to 0.7 % of its initial value.

In continuing the investigation of the action of proteolytic enzymes, attention was then turned to those of animal origin. It was stated by Dudley [1919] and by Thorpe [1926] that pancreatic trypsin destroys the hormone. Dale and Dudley [1921] also recorded a destruction by intestinal erepsin. Freudenberg *et al.* [1932] found that pepsin and erepsin did not attack the hormone, but that it was destroyed by trypsin² and also by papain. No details of the nature of the erepsin used were given by these latter authors, but in view of the date of the investigation it may be presumed to have been more nearly enzymically homogeneous than that of Dale and Dudley.

Apparently on the basis of these results, Freudenberg and his collaborators concluded that the hormone has either a high molecular weight or is bound to a high-molecular carrier of the protein group. They also stated that the hormone is evidently of the same type as insulin. These views are in opposition to the conclusions of Smith and McClosky [1924] and of Kamm [1928] which were based on comparisons of rates of dialysis and on the relative permeability of the membranes used to the hormone, adrenaline and dyestuffs. Smith and McClosky observed that the diffusibility of the hormone was about the same as that of methylene blue, and Kamm suggested 600 as the molecular weight. He also concluded that the oxytocic hormone is more complicated than adrenaline, but simpler than insulin or the parathyroid hormone.

Unpublished experiments of our own on the pressure dialysis through pyroxylin thimbles of solutions of the hormone which had undergone a purification by charcoal adsorption [Gulland and Newton, 1932] led to the view that the hormone is probably not proteinoid in character. Consequently the destruction of the hormone by trypsin preparations was probably not due to trypsin

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² It is assumed that activated trypsin was intended, since Waldschmidt-Leitz and Purr [1929] have shown that trypsinogen (unactivated trypsin) has no proteolytic activity.

itself, as believed by the investigators mentioned above, but was the result of one or more accompanying enzymes. In arriving at this conclusion we were also influenced by the observation in Part III that the inactivation by papain preparations, which was quoted by Freudenberg *et al.* [1932] in support of the protein nature of the hormone, is in fact due to another enzyme, which is not a papainase and has no action on gelatin.

When this conclusion was investigated experimentally, it was found that the hormone was extremely rapidly inactivated by a trypsin preparation supplied by British Drug Houses, Ltd. The shape of the p_H -activity curve and the position of the p_H optimum (Fig. 1) resembled in general those which characterise the action of activated trypsin on proteins. Markedly different results,

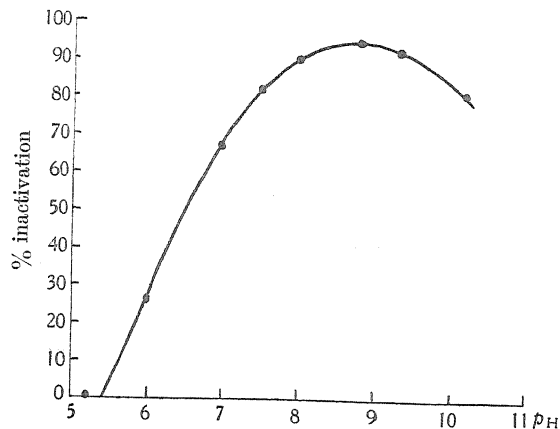


Fig. 1. p_H -activity curve of inactivation by trypsin preparation.

however, were obtained with the same weight of an 11-year old Kahlbaum preparation of trypsin under the same conditions; the hormone was inactivated, but only very slowly. The possession of these widely different trypsin preparations offered the opportunity of comparing directly their tryptic activity with their power of inactivating the hormone, and thus of determining whether activated trypsin itself has any action on the hormone.

By following with the titration method the early stages of the hydrolysis of gelatin by these enzyme preparations, it was shown (Table III) that the tryptic activity of the B.D.H. preparation was about 50 times stronger than that of the Kahlbaum preparation; by considering only the early stages of the hydrolysis it was permissible to discount for practical purposes the effects due to creptic activity, which also was considerably higher in the B.D.H. preparation.

Two parallel experiments under identical conditions were then carried out to compare the powers of inactivating the hormone of the B.D.H. trypsin preparation on the one hand and of 50 times its weight of the Kahlbaum preparation on the other. If activated trypsin is responsible for inactivating the hormone, the inactivation in these two experiments should have been about the same. In actual fact, however, the inactivation by the B.D.H. preparation was many times more rapid than by the Kahlbaum preparation (Fig. 2). Thus activated trypsin itself is not responsible for the inactivation of the hormone by trypsin preparations, but this is caused by an enzyme with a p_H optimum at 8.8 (Fig. 1). This enzyme is not identical with the inactivating enzyme of yeast

and papain (Part III), and it is improbable, though not quite excluded, that they are the plant and animal variants of the same type of enzyme (identical substrates), because the enzyme of the trypsin preparation reduced the oxytomic value to 0.3 % (Table I) as compared with the value of 0.7 % in the experiments with the yeast-papain enzyme. As in the case of the activity remaining after the action of the latter enzyme, our present opinion is that the residual activity of 0.3 % is due to a derivative of the hormone produced by enzymic action.

It has thus been shown in this publication and in Part III that the apparent inactivation of the hormone by enzymes of which the characteristic substrates are proteins is not brought about by those enzymes themselves but by unidentified enzymes which accompany them. These facts seem to dispose of the contention that the hormone is proteinoid in character.

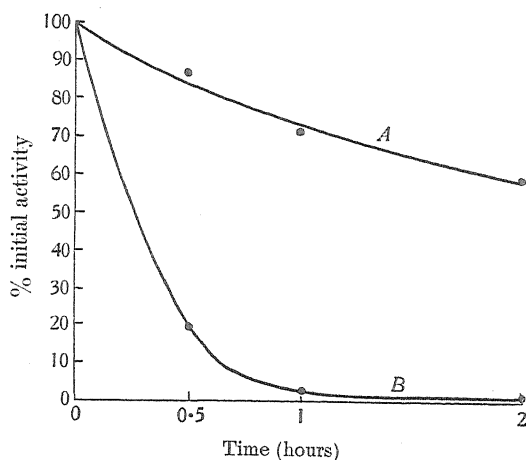


Fig. 2. Comparison of inactivation by weights of Kahlbaum trypsin (A) and B.D.H. trypsin (B) preparations having the same tryptic activity.

From the same experiments it is apparent that the sole support for the view advanced by others that the hormone is peptide in character lies in the fact that the enzymes which inactivate it accompany proteolytic enzymes. We are not opposed to the conception that the hormone molecule contains peptide linkages, but at present there is no evidence on this point.

Freudenberg *et al.* [1932] observed that pepsin did not attack the hormone. Presumably, although this is not stated, their experiments were made at or about the strongly acid p_H optimum of pepsin. As the beginning of a general study of the effect on the hormone of the enzymes of animal tissues, a proteolytically potent specimen of pepsin was examined at p_H 4.4 and p_H 7.2 for the presence of other enzymes which might attack the hormone. In neither case was any diminution in oxytomic strength observed. A bone phosphatase preparation also was tested at p_H 7.8 and found to be quite inactive towards the hormone.

From what has already been stated and from a general consideration of the problem, it is evident that a series of favourable results in a definite and somewhat prolonged sequence of investigations is required for the complete experimental demonstration that any given enzyme does inactivate the hormone. In the proof that an enzyme does not inactivate the hormone, a part or even most

of this sequence may be omitted. Apart from haphazard guesswork, the suggestion that a particular well-defined enzyme inactivates the hormone must be formulated as a result of facts observed in one or more of the following modes of attack: from a systematic examination of the effects of enzymes in general; from predictions of the presence of chemical groups as a result of the study of the action of chemical reagents; from the similarity of the p_H -activity curves of the inactivating enzyme acting on the hormone and of the suspected enzyme¹ acting on its known substrate. The last-mentioned source suffers from the disadvantage that the shape and optimum of a p_H -activity curve may vary with change of substrate. The stages of the demonstration are tabulated below.

(1) The discovery of the rate at which the hormone is inactivated by the preparation of the inactivating enzyme, and the determination of the p_H -activity curve.

(2) The examination of the action of the inactivating enzyme on the substrate of the suspected enzyme. If the degree of activity permits, the determination of the p_H -activity curve.

(3) The examination of the action of an authentic preparation of the suspected enzyme on the hormone, including the determination of the rate of action and of the p_H -activity curve for comparison with those in (1).

(4) Careful estimations of the activities of (a) an authentic preparation of the suspected enzyme, and (b) of the inactivating enzyme preparation towards the substrate of the suspected enzyme. This will permit a numerical comparison of the amounts of the suspected enzyme present in its authentic preparation and in the preparation of the inactivating enzyme.

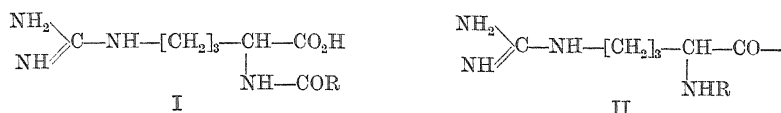
(5) The demonstration of equal inactivation of the hormone under the same conditions by weights of preparations of the suspected enzyme and inactivating enzyme, which it is estimated would cause the same degree of inactivation if the suspected enzyme and inactivating enzyme were identical.

It was shown in Part II [Gulland, 1933] that two out of the three well-defined inactivations of the hormone which occur in presence of nitrous acid are probably due to nitrous acid itself. The rates of these inactivations and the conditions under which they occurred were not inconsistent with the view that the slower, which required a large excess of the reagent, was due to the interaction of nitrous acid with a guanidine group, and that the more rapid was the result of its interaction with an amino- or basic imino-group. A preliminary consideration from the enzymic aspect was not opposed to this conception, which was therefore examined more fully.

The position at a relatively alkaline reaction of the p_H optimum of the inactivation of the hormone by the B.D.H. trypsin preparation was not far removed from that of arginase. Moreover, this preparation contained a small, but definite, arginase activity, which was estimated by a modification of the arginase-urease method of Edlbacher and Röthler [1925]. In accordance with the procedure outlined above for the examination of a suspected enzyme, a highly potent glycerol solution of arginase was prepared from fresh liver [Edlbacher and Röthler, 1925]. The amounts of this arginase solution and of the B.D.H. trypsin preparation were then calculated from their arginase activities, so that, if arginase were the inactivating enzyme, the arginase solution should have caused approximately twice as much inactivation of the hormone as the trypsin preparation—twice as much, rather than the same, inactivation,

¹ This term denotes the well-defined enzyme with known substrate which is suspected of attacking the hormone. The term "inactivating enzyme" denotes the unknown enzyme in a preparation which attacks the hormone.

in order to emphasise the result. When these enzymes were examined under identical conditions for their action on the hormone, the trypsin preparation reduced the activity to 7 % of its initial value in 30 minutes, whereas with the liver extract no inactivation was detectable after 4 hours. Thus the inactivating enzyme of the B.D.H. trypsin preparation is not arginase. The papain preparation used in the earlier experiments exhibited no arginase activity, and hence the hormone does not contain the group I, although the group II or another guanidine derivative is not excluded [Felix *et al.*, 1928; Edlbacher and Burchard, 1931].



The shape and position of the optimum of the p_{H} -activity curve of the inactivating enzyme of the yeast and papain preparations (Part III) closely resembled those (Fig. 3) of prolinase, of which the simplest substrate is prolylglycine [Grassmann *et al.*, 1932]; the substrates of this enzyme all contain the

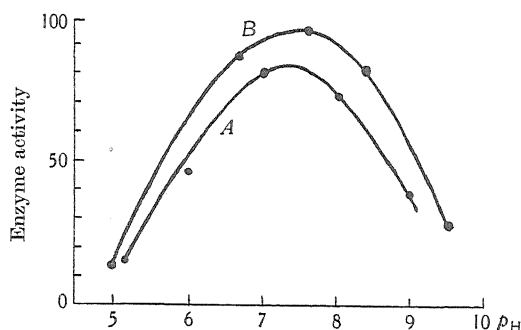


Fig. 3. p_{H} -activity curves of inactivating enzyme of yeast proteinase solutions (A) and of Grassmann's prolinase (B).

basic imino-group of proline. When the yeast dipeptidase, yeast proteinase and papain preparations used in Part III were tested for prolinase activity, all contained traces of this enzyme. It seemed probable that these amounts were too small for prolinase to be the inactivator, but, on the other hand, the affinity of the inactivating enzyme for the hormone is unknown and may be very great. The routine examination already described was therefore undertaken. A solution of prolinase, prepared from a neutral autolysate of yeast, contained more than 50 times the prolinase activity of the yeast proteinase solution. From this figure, a calculation was made of the amounts of the two enzymes needed to compare their power of inactivating the hormone; as before, however, only half the requisite amount of proteinase preparation was used, in order to emphasise the result. The enzyme of the proteinase solution reduced the oxytocic value to 38 % in 30 minutes, whereas with the yeast autolysate it had only become 90 % in that time, and after 4 hours was still 73 %. Thus, the inactivating enzyme of the yeast and papain preparations is not prolinase.

The B.D.H. trypsin preparation contained moderate amounts of an enzyme which hydrolysed prolylglycine. The dependence of the activity of this enzyme

on hydrogen ion concentration resembled that of prolinase [Grassmann *et al.*, 1932] in having an optimum just below p_H 8. It was therefore improbable that the inactivating enzyme (optimum at p_H 8.8) of the B.D.H. trypsin preparation was prolinase.

EXPERIMENTAL.

As in previous communications, the oxytocic activity of solutions was assayed by the degree of contraction they produced in the isolated uterus of the guinea-pig. The technique described in Part III was adopted in the present experiments.

Action of B.D.H. trypsin preparation at p_H 7.5.

A hormone solution (2.0 cc., containing 156 oxytocic units per cc.), $M/3$ sodium phosphate at p_H 7.5 (3 cc.) and a solution of B.D.H. trypsin preparation (10 mg. in 2 cc. of water) were mixed, and the mixture diluted to 10.0 cc. at p_H 7.5 by means of water and a trace of N ammonia. A control sample (1.0 cc.) was immediately removed into a 2.5 cc. flask, heated for 3 minutes in a boiling water-bath and placed in a thermostat at 40°. The remainder of the mixture had meantime been placed in the thermostat. Further samples were removed at appropriate intervals, and finally all were diluted to 2.5 cc. at p_H 3.5 with 2.5 N acetic acid (0.5 cc.) and water. Each sample was assayed against the control solution, 0.2 cc. being diluted to 10 cc. at p_H 7.5; these dilutions would therefore have contained 0.25 unit per cc. if no inactivation had occurred. The results are given in Table I.

Table I.

Time (hours)	0.5	1	6	27
Activity present (%)	0.7	0.5	0.3	0.3

Action of Kahlbaum's trypsin at p_H 7.5.

The amounts of hormone, enzyme and buffer were those of the preceding experiment, and the procedure was exactly the same (Table II).

Table II.

Time (hours)	1	6	24
Activity present (%)	100	76	40

Comparison of the action of B.D.H. and Kahlbaum trypsin preparations.

A. *Proteolytic activity.* The proteolytic activities of the two preparations were compared by the method of titration in 90 % alcohol with alkali, gelatin being used as substrate. Gelatin solution (6.0 cc. of 10 %), $M/3$ sodium phosphate buffer at p_H 7.5 (1.0 cc.) and varied amounts of the solid enzyme preparations were mixed and diluted to 10.0 cc. at p_H 7.5, the p_H being adjusted with ammonia or acetic acid. In each case a sample (1.0 cc.) was immediately removed into 9 cc. of absolute alcohol at 60°, this mixture being titrated with $N/20$ alcoholic KOH whilst being shaken. The reaction mixtures were incubated at 40°, and further samples were taken at the times stated in Table III.

It is evident (see especially columns marked †) that the proteinase activity of the B.D.H. preparation was about 50 times greater than that of the Kahlbaum preparation. By comparing only the increases in acidity in the early stages of the hydrolysis, the effects due to ereptic action may be disregarded or estimated. Of the two preparations, the B.D.H. one is richer in ereptic activity; this is shown by the greater increase in acidity in the later stages of the experiments in which it is used.

Table III.

The figures denote increases in acidity of 1 cc. portions of the reaction mixtures, expressed as cc. of $N/20$ KOH. The weight of the enzyme preparation used is shown above each column.

Kahlbaum trypsin prep. (Dec. 1922)				Time hours	B.D.H. trypsin prep. (May 1931)			
10 mg.*	20 mg.	100 mg.	500 mg.†		1 mg.	2 mg.	10 mg.	10 mg.†
—	—	—	0.38	$\frac{1}{4}$	—	—	—	0.38
—	—	—	0.68	$\frac{1}{2}$	—	—	0.65	0.74
—	—	0.38	0.76	1	0.23	0.45	—	0.96
0.05	—	0.46	—	2	0.40	0.53	—	—
—	—	—	—	5	—	—	1.14	—
0.15	—	—	—	$5\frac{1}{2}$	—	—	—	—
—	—	0.60	—	6	0.82	0.88	—	—
—	0.47	—	—	17	—	—	—	—
—	—	—	—	20	—	—	1.48	—
0.52	—	—	—	23	—	—	—	—
—	0.51	—	—	41	—	—	—	—
0.60	—	—	—	47	—	—	—	—

* The reaction mixture contained 1.0 cc. of $N/2.5$ ammonium chloride in addition to the other components.

B. *Inactivation of the hormone.* The experiments with both enzymes were made under identical conditions. A hormone solution (1.0 cc., containing 156 units), $M/3$ sodium phosphate buffer at p_H 7.5 (1.5 cc.) and a solution of the enzyme preparation (125 mg. of Kahlbaum trypsin, or 2.5 mg. of B.D.H. trypsin) in water (0.5 cc.) were mixed, adjusted to p_H 7.5 with N ammonia and diluted to 5.0 cc. with water. The customary procedure was observed, the samples measuring 1.0 cc. and being taken at the same intervals. The samples were assayed against their control samples, 0.2 cc. in each case being diluted to 10 cc. at p_H 7.5. The results are given in Table IV and Fig. 2.

Table IV.

Kahlbaum prep., activity present (%)	Time hours	B.D.H. prep., activity present (%)
86	$\frac{1}{2}$	19
71	1	2.7
59	2	0.6

These results showed that the inactivation of the hormone was not caused by trypsin, assuming that the different rates of inactivation were not due to a more rapid destruction of the trypsin in one preparation than in the other. It was shown that if destruction did occur, it was approximately equal in the case of both enzymes. At the end of the incubations the residual proteinase activity was determined as follows. Each enzyme-hormone-buffer mixture (1 cc.) was mixed with 10 % gelatin solution (3 cc.), and the mixture was adjusted to p_H 7.5, diluted to 5 cc. with water and incubated at 40°. The rate of liquefaction of the gelatin was then followed. After 1 hour the gelatin of both mixtures solidified with great difficulty when cooled under the tap, the times required for solidification being approximately the same. After 2 hours the gelatin of both experiments did not solidify.

Effect of hydrogen ion concentration on the rate of inactivation of the hormone by the B.D.H. trypsin preparation.

An aqueous solution of B.D.H. trypsin (1.25 mg. in 0.25 cc.) was added to an ice-cold mixture of a hormone solution (0.5 cc., containing 156 units per cc.) and $M/3$ sodium phosphate buffer at the required p_H (1.75 cc.). A control sample was immediately removed into a 2.5 cc. flask, heated for 3 minutes at 100° and placed in the thermostat at 40° . The remainder of the solution was kept in the thermostat for 30 minutes, when a second sample was taken as described above. The p_H of the remainder of the solution was determined and taken as the p_H of the experiment; determinations at p_H 9.3 and 10.2 were made with a hydrogen electrode, the others colorimetrically.

The experimental solutions were assayed against the control solutions, 0.2 cc. in each case being diluted to 10 cc. at p_H 7.5. The results are given in Table V and Fig. 1.

Table V.

p_H	5.2	6.0	7.0	7.5	8.0	8.8	9.3	10.2
Inactivation (%)	0	26	67	82.5	90	95	92.5	81

Action of B.D.H. pepsin preparation.

A. At p_H 7.2. A hormone solution (6.7 cc., containing 3 units per cc.), $M/3$ sodium phosphate solution at p_H 7.5 (1 cc.) and a solution of active B.D.H. pepsin (100 mg. in 2 cc. of water) were mixed and diluted to 10 cc. at p_H 7.2. A control sample (2.0 cc.) was immediately removed, and other samples were taken at $\frac{1}{2}$, 2, and 25 hours. The usual technique was followed.

B. At p_H 4.4. The procedure was exactly as described above, except that N acetate buffer was used. The p_H of the mixture was 4.4. In both series of experiments the samples were assayed against the controls, 1 cc. being diluted to 10 cc. In no case could any inactivation be detected.

Action of bone phosphatase. A hormone solution (0.25 cc., containing 312 units per cc.), $M/5$ sodium phosphate at p_H 7.8 (1 cc.) and a solution of a bone phosphatase preparation (10 mg. of $A/W=0.23$ [Martland and Robison, 1929]) in water (1 cc.) were mixed and diluted to 5 cc. with water. The p_H was 7.8. The usual technique was followed, samples (1 cc.) being removed at $\frac{1}{2}$, $1\frac{1}{2}$, and 26 hours and assayed against the control sample. No inactivation of the hormone was detected.

Consideration of the possibility that arginase inactivates the hormone.

A. *Estimation of arginase in B.D.H. trypsin preparation.* A solution of trypsin (100 mg.) in water (2 cc.), $M/10$ glycine-sodium hydroxide buffer at p_H 9.5 (2 cc.) and a 1% d -arginine solution (2 cc.) were mixed, and the mixture was adjusted to p_H 9.5, incubated for 1 hour at 40° and heated for 4 minutes at 100° . The p_H of the mixture was then adjusted to 7, and a suspension of one powdered tablet of B.D.H. urease¹ in $M/3$ phosphate buffer at p_H 7 (1 cc.) was added. The mixture, in a closed tube, was incubated at 40° for 1 hour, cooled and transferred to a micro-Kjeldahl apparatus containing saturated sodium carbonate solution (2 cc.) and some medicinal paraffin (to prevent frothing). Steam was passed into the apparatus in the usual way², and the

¹ One tablet hydrolyses 50 mg. urea in 1 hour at 35° .

² This is a modification of the method of Edlbacher and Röthler [1925]. They aspirated the ammonia from the warm solution, but we obtained inconsistent results owing to incomplete removal of ammonia.

distillate was collected in *N*/50 hydrochloric acid (10.0 cc.); after actual distillation had proceeded for 3 minutes, the contents of the receiver were titrated to methyl red with *N*/50 NaOH. Control experiments were also carried out following the same procedure, except that the trypsin-buffer-arginase mixture was heated for 4 minutes before incubation instead of afterwards. The experiments were repeated several times, and in all cases a small but definite arginase activity was detected. It was estimated that under the conditions of the experiment 100 mg. of the trypsin preparation yielded 0.10–0.15 cc. of *N*/50 ammonia.

B. *Estimation of arginase activity in a preparation from liver.* The arginase content of a glycerol extract [Edlbacher and Röthler, 1925] of guinea-pig liver was estimated as described above. The 1:3 glycerol extract (1 cc.) was diluted to 10 cc., and 0.2 and 0.4 cc. of the dilution used for the estimation (Table VI).

Table VI.

Diluted glycerol extract (cc.)	Experiment cc. <i>N</i> /50 NaOH	Control cc. <i>N</i> /50 NaOH	Ammonia formed cc. <i>N</i> /50
0.2	7.48	9.69	2.21
0.4	6.08	9.65	3.57

C. *Comparison of hormonal inactivation by B.D.H. trypsin and arginase preparations.* The two experiments followed the customary procedure of assaying experimental against control solutions and need not be described in detail. The volumes of the reaction solutions were 2.5 cc., the p_H values were 9.3, and the amounts of hormone used were comparable. In one experiment the B.D.H. trypsin preparation (1.25 mg.) was used, in the other a solution containing 0.00004 cc. of the glycerol extract of guinea-pig liver. This solution thus contained about twice as much arginase activity as the amount of trypsin preparation used. The trypsin preparation effected 92.5 % of the possible inactivation in $\frac{1}{2}$ hour, whereas little or no inactivation (possibly as much as 10 %) occurred after 4 hours in the experiment with arginase solution. If arginase inactivated the hormone, the solution of the glycerol extract of arginase should have caused about twice as much inactivation as the trypsin preparation. It follows, therefore, that the inactivating enzyme of the trypsin preparation was not arginase.

The B.D.H. papain preparation contained no arginase, nor did it exhibit any urease activity, which would have interfered with the arginase estimation. The inactivating enzyme of yeast and papain preparations is not arginase.

Consideration of the possibility that prolinase inactivates the hormone.

A. *Estimation of prolinase in preparations of yeast proteolytic enzymes.* Prolinase activity was measured by titrating the increased acidity of prolylglycine with alkali in 90 % alcohol [Grassmann *et al.*, 1932]. *dl*-Prolylglycine (40 mg.) was dissolved in *M*/3 phosphate buffer at p_H 7.5 (0.25 cc.) in a 2.5 cc. flask. An aqueous solution (1.5 cc.) of a yeast dipeptidase, yeast proteinase or B.D.H. papain preparation was added, and the mixture was diluted with water and alkali to 2.5 cc. at p_H 7.5. The enzyme preparations were those used in Part III. A sample (0.5 cc.) was immediately removed into a flask containing absolute alcohol (4.5 cc.) and titrated to thymolphthalein with *N*/20 potassium hydroxide in 90 % alcohol. The remainder of the solution was incubated at 40°, and samples were taken at 4 and 23 hours. In the control experiment the enzyme solution was replaced by water. The results are given in Table VII, and it will be seen that each enzyme preparation contains traces of prolinase.

Table VII.

The figures represent the degree of hydrolysis and are the increase in acidity of 0.5 cc. samples quoted as cc. *N*/20 alcoholic KOH.

Time hours	Control	Dipeptidase 5 mg.	Papain 5 mg.	Proteinase 0.5 cc.
4	0.00	0.01	0.02	0.01
23	0.00	0.06	0.04	0.02

B. *Preparation of a potent prolinase solution from yeast.* English brewer's yeast (50 g.) was liquefied with ethyl acetate (5 cc.) and allowed to autolyse for 1.5 hours at p_H 6.5–7, ammonia being added to maintain the p_H . The yeast was centrifuged, washed and then allowed to autolyse at p_H 7.0 for 24 hours in presence of a few drops of toluene. Finally the yeast was centrifuged, and the prolinase activity of the clear solution was estimated as described above. Acting on *dl*-prolylglycine (40 mg.) for 2 hours at p_H 7.5 and 40° in a total volume of 2.5 cc., the prolinase solution (0.5 cc.) caused an increase in the acidity of 1.0 cc. portions corresponding to 0.24 cc. of *N*/20 KOH. The prolinase solution had therefore more than 50 times the prolinase activity of the proteinase solution (above).

C. *Comparison of hormonal inactivation by yeast proteinase and prolinase solutions.* Hormone solutions (each containing 30 units) were mixed either with the proteinase solution (0.25 cc.) or with an aqueous dilution of the yeast autolysate corresponding with 0.007 cc. of this autolysate; the experiment with the proteinase thus contained about half the prolinase activity of that with the yeast autolysate. The mixtures were diluted to 2.5 cc. at p_H 7.0, being buffered with *M*/15 sodium phosphate, and were incubated at 40°. The usual procedure was followed, samples being removed immediately and after 30 minutes; a sample from the yeast autolysate experiment was also taken after 4 hours. In the proteinase experiment 38 % of the initial oxytocic activity remained after 30 minutes, whereas in the yeast autolysate experiment more than 90 % was present after that period, and after 4 hours 73 % still remained. The inactivating enzyme of the yeast and papain preparations is therefore not prolinase.

D. *Action of B.D.H. trypsin preparation on prolylglycine.* *dl*-Prolylglycine (40 mg.) in *M*/3 phosphate buffer at the appropriate reaction (0.25 cc.) was mixed with a solution of B.D.H. trypsin preparation (25 mg.) in water (0.5 cc.) in a 2.5 cc. flask. The mixture was adjusted to the required p_H and diluted to 2.5 cc. Samples (1.0 cc.) were removed for titration in the usual way at the beginning and after 2½ hours. The control experiments contained no substrate. The results are given in Table VIII.

Table VIII.

The figures represent increases in acidity in terms of *N*/20 KOH.

p_H	6.0	7.0	8.0	9.0
Experiment	0.16	0.27	0.44	0.29
Control	0.06	0.07	0.12	0.16
Due to prolinase	0.10	0.20	0.32	0.13

Difficulties encountered in determining the end-points increased the experimental error of these results. It is evident however that the p_H optimum of the prolinase is considerably less alkaline than p_H 9 and probably less than p_H 8. The inactivating enzyme of the trypsin preparation, having an optimum at p_H 8.8, is probably not prolinase.

An experiment similar to that described above was carried out with the Kahlbaum trypsin preparation (125 mg. in 2.5 cc.) at p_H 7.2. No increase in acidity was observed after incubation for 2.5 hours. This preparation, therefore, did not contain prolinase.

SUMMARY.

1. A B.D.H. trypsin preparation inactivated the hormone very rapidly at p_H 7.5, the p_H optimum of the inactivation being 8.8.
2. Under similar conditions a Kahlbaum trypsin preparation inactivated the hormone extremely slowly.
3. Experiments in which quantities of these preparations having equal tryptic activities acted on the hormone showed that, contrary to existing conclusions, the inactivating enzyme was not trypsin. This result, conjoined to the earlier proof that the inactivating enzyme of papain preparations is not a papainase, affords strong evidence against the contention that the hormone is proteinoid.
4. In attempts to identify the inactivating enzyme of yeast and papain preparations and also that of the B.D.H. trypsin preparation, it was shown that neither arginase nor prolinase inactivates the hormone.
5. No evidence is yet available to support the view that the hormone contains peptide linkages.
6. The hormone was not attacked by a B.D.H. pepsin preparation at p_H 4.4 or at p_H 7.2, or by a bone phosphatase preparation at p_H 7.8.

Our thanks are due to Messrs Boots Pure Drug Company, Nottingham, who generously presented the posterior lobe powder used in this work.

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CLXXXVIII. THE QUANTITATIVE EXTRACTION OF HISTAMINE FROM TISSUES BY ELECTRODIALYSIS.

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(Received July 12th, 1933.)

DURING an investigation involving a large number of histamine assays, it occurred to us that the chemical manipulation could be greatly simplified if the base could be extracted by electrodialysis. A further advantage of the method would be the elimination of all but basic substances of low molecular weight from the extract. In the method we had been using [Best *et al.*, 1927] the tissue is extracted with 96 % alcohol for 24 hours, the alcohol removed and the meat re-extracted with 60 % alcohol for a further 24 hours. The combined extracts are concentrated *in vacuo* and fat removed by extraction with ether. The extracts so prepared were usually yellow and often slightly cloudy, although free from biuret products.

By electrodialysis in a three compartment cell a water-clear colourless extract is obtained in less than 2 hours after receipt of the tissue: this means a great saving of time when many samples have to be worked up. In a large number of experiments during the last 3 years we have never found an electrodialysate giving a smaller histamine value than an equivalent dose of an extract prepared by the alcohol method. (The histamine value of the extracts was obtained by matching against a standard of pure histamine on the blood pressure of an anaesthetised cat.) Further, histamine added to blood, which contains no histamine, has been repeatedly extracted without loss. Histamine added to tissues which contain histamine has also been recovered without detectable loss.

The process of electrodialysis does not subject the tissues in the middle compartment to any drastic conditions. The temperature does not rise above 40°, and there is no great change in the p_H of the liquid in the middle compartment. The method therefore supports the alcohol method not only quantitatively but also in the suggestion that the histamine in the tissues is, if not free, very loosely combined.

On a few occasions we have also extracted a portion of the tissue by the more drastic method of Best and McHenry [1930], in which the tissue is heated with 10 % HCl at 95° for 1 hour and then evaporated to dryness *in vacuo*; alcohol is added and distilled off to remove some of the acid, and the residue is taken up in water, neutralised and filtered. These extracts, which are clear but dark brown in colour, usually give a value about 30 % higher than extracts prepared by either of the above methods. In view of the agreement between the alcohol and dialysis methods and our quantitative recovery of added histamine, we feel that it is highly significant that extracts prepared by the Best and McHenry method give a strong biuret and several other protein reactions

not given by the alcohol extracts or electrodialysates of the same tissue; especially since we have observed an increased histamine value when a membrane passed biuret products.

It might be argued that the lower value obtained by the dialysis is due to loss of histamine by autolysis [Best, 1929] in the dialysis cell, whilst the lower value by the alcohol method is due to incomplete extraction. Apart from the coincidence of the errors, it should be noted that the time for which the tissue is in the region of 37° rarely exceeds half an hour. This short time would not be sufficient, judging from Best's curve, to cause any perceptible loss in histamine. Again, Best *et al.* [1927] were unable to detect any difference in the histamine content of a dog's lungs worked up immediately after removal from the animal or after keeping 2 hours; and MacGregor and Peat [1931] found no change in the histamine in a lung mince which had stood overnight.

EXPERIMENTAL.

Description of apparatus.

Cells. The cells used for these experiments were, with one exception, made from wood to the pattern of those of Foster and Schmidt [1923]. Small cells of 100 cc. capacity in each compartment were carved from solid blocks of well-seasoned beech or Oregon pine. Larger cells of 250 cc. capacity (Foster and Schmidt size) were built up from pieces of thick wood. The wood was well impregnated with high-melting paraffin wax. The waxing is of some importance since a badly waxed cell often gives up during dialysis an alkali-soluble substance giving a strong Pauly reaction. This substance is insoluble in neutral or acid solution. Still larger cells used in some of the preliminary experiments were made from three glass museum jars, faces $7" \times 5\frac{1}{2}"$, with $3\frac{1}{2}"$ holes cut in the appropriate faces. The capacity of each compartment varied from 600 cc. to 1500 cc. according to the width of the jars used. For the wooden cells the gaskets were made of ordinary red rubber sheet $\frac{1}{16}"$ thick. Owing to their fragility the glass cells required special soft rubber gaskets $\frac{3}{8}"$ thick. These were specially made for us by Messrs C. Macintosh and Co., Ltd., Manchester.

Electrodes. The cathodes were made from pure nickel sheet slightly smaller than the cross section of the cell. The anodes were made from one or two $6" \times 2" \times \frac{1}{4}"$ carbon plates according to the size of the cell.

Membranes. In the experiments with lung and blood ordinary parchment dialysing-paper or cellophane sheets were found quite satisfactory. With liver and heart, especially the former, these membranes passed traces of "biuret" products, and the dialysates gave histamine values perceptibly higher than those obtained from the corresponding alcohol extracts. The passage of such substances is prevented by using cathode membranes of collodion. We found it simpler to make sacs by the inside tube method in $10" \times 1\frac{1}{2}"$ test-tubes than to make sheets direct by the plate or mercury method. The sacs were then cut up into sheets and kept in distilled water until required. 14 % pyroxylin in equal parts (by weight) of ether and absolute alcohol [Walpole, 1915] gave sufficiently robust membranes.

Stirring. The liquid in the middle compartment was kept stirred mechanically to prevent any local aggregation of mince. Some tissues, especially heart muscle, tend to form a layer on the cathode membrane which increases somewhat the electrical resistance of the cell and thus slightly increases the time required for dialysis. This layer was broken up from time to time by a glass rod.

Current. The cell was connected to 230 volt D.C. mains in series with a resistance of a bank of 4 carbon filament lamps in parallel. Each lamp passed about 1 amp. and could be switched into or out of the circuit as required. For the smaller cells 2 or 3 lamps were generally sufficient. The mean consumption of the 100 cc. cells was about 50 watt-hours, and of the 250 cc. cell about 120 watt-hours.

Temperature. The temperature was kept down by providing each compartment with glass cooling coils supplied with a rapid stream of cold water. In this way the temperature even in the smallest cell could be kept below 40° when the current passing was 1 amp. The temperature only reached this value while a heavy current was passing. This rarely occurred for more than half an hour. A typical curve showing the current and temperature changes is given in Fig. 1.

p_H . The p_H change in the middle compartment was not sufficient to warrant alteration, e.g. by addition of baryta. With tissues, the p_H in the middle compartment, which is initially just above 7, usually falls to about 4.6. We have never observed a p_H more acid than this. With blood, the p_H does not usually fall below 6.

End-point. Theoretically, when the resistance of the cell has attained a steady value and the titratable alkalinity is constant, all the histamine should have passed to the cathode chamber. In view, however, of the difficulty of accurate current measurement without special instruments and the minute amount of histamine present relative to other electrolytes, we found it more reliable to use the Pauly reaction as an indication of the completion of the dialysis. Samples were withdrawn from the cathode chamber at 15 minute intervals until two successive samples gave the same colour. For this purpose we have adapted Gebauer-Fülneegg's [1930] modification of the reaction as a quantitative method. 1 cc. of test solution is mixed with 1 cc. $N/2$ Na_2CO_3 and to this are added 2 cc. of fresh diazo-reagent prepared by mixing 1 cc. of 0.125 % *p*-nitraniline in $N/10$ HCl with 1 cc. 0.37 % $NaNO_2$. The colour gradually deepens and then fades, finally becoming cloudy. The maximum colour intensity, which usually appears in about 1 minute, is recorded in red and yellow units in a Rosenheim and Schuster [1927] colorimeter. Concentrations from 1/33,000 to 1/300,000 can be read off directly without dilution. Plotting colorimeter red units against histamine concentration gives an approximate straight line over this range. The method gives consistent results and for our purpose is quicker and more convenient than the Koessler and Hanke [1919] method which we used in our early experiments. But it must be stressed that this, like other adaptations of the Pauly reaction, is not specific for histamine and cannot be used for the estimation of the base in tissue extracts. In every case our Pauly values have been higher than would be expected from the blood pressure assay. The reaction could be used as an indicator for the extraction from tissues of any alkali-stable base which passes to the cathode with the same ease as histamine.

Since the convenience of this end-point depends upon the use of a Rosenheim and Schuster colorimeter, we have studied the current relations throughout the experiments with a view to giving an alternative method for determination of the end-point. Measurements of the voltage across the cell and amperage of the circuit were noted every 15 minutes. From the area of a watt-time curve drawn from these data the total consumption in watts was obtained. In 60 experiments with 4 different cells we have found the ratio WT/ad to be approximately constant for a given tissue. T is the time taken to attain a constant

Pauly value, a the area of the cathode in cm. and d the distance in cm. of the cathode from the centre of the middle compartment. The mean values of this ratio are given in Table I. (In only 5 cases were the values more than ± 0.1 from the mean.) As would be expected these values are roughly proportional to the mean values for the titratable alkalinity of the cathode liquids. The latter values, expressed as cc. N acid per 100 cc. of dialysate, and the weight of tissue corresponding to this volume are also included in the table.

Table I.

Tissue	$\frac{WT}{ad}$	Titratable alkalinity cc. N acid	Weight of tissue g.	Number of experiments
Blood	0.63	3.9	30	15
Heart-muscle	0.49*	2.6	20	18
Liver	0.43	2.6	20	11
Lung	0.53	3.3	20	16

* This value is probably relatively high, since with heart-muscle there was a much greater accumulation of tissue on the cathode membrane than with any other tissues examined.

Since we had no indication in any experiment that prolonging the dialysis caused any loss of histamine, it should be safe to assume the complete passage of any equally stable and dialysable base when sufficient current has been passed to satisfy the equation $WT/ad = 0.6$ for the tissues or $WT/ad = 0.7$ for blood.

Method.

The cell having been assembled, the cathode and anode compartments are filled to capacity, say 250 cc., with distilled water. The middle compartment is filled with 200 cc. distilled water and 50 g. of the minced tissue. It is not necessary to add an electrolyte to the distilled water. After turning on the cooling water and starting the stirrer, the current is switched on. If the small cells are used, the current and temperature in the middle compartment, which rise rapidly at first, fall after about 1 hour. The end-point is then determined as already described (p. 1396). The cathode liquid is then removed and the chamber washed out. The combined liquid and washings after neutralisation with $N H_2SO_4$ and adjustment to appropriate volume are ready for the physiological assay.

Physiological assay. The extracts were compared as to their depressor effect when injected into the femoral vein of an anaesthetised cat. At least two definite matches were obtained before the pairs of extracts were recorded as equal. Histamine values were obtained by matching against a standard histamine solution [Best *et al.*, 1927; Burn, 1928]. The cats were anaesthetised with medinal (0.42 g. per kg. body weight) after induction with ether.

RESULTS.

Comparison of alcohol and electrodialysis methods. Three histamine-containing tissues were examined. In all the experiments the tissue was minced and well mixed. Samples were then worked up by the dialysis method and the alcohol method and assayed physiologically. There was no detectable difference. These experiments are summarised in Table II, which also shows the extreme histamine values for each tissue.

Table II.

Tissue	Source	Number of experiments	Extreme histamine values in mg. per kg.
Heart (ventricular muscle)	Ox	8	8-25
Liver	Dog	2	13-40
	Ox	2	10-20
	Pig	2	13
	Sheep	3	2.5-6
Lung	Dog	3	10-13
	Ox	11	30-60

In the case of the liver which contains choline, the dialysates and the alcohol extracts were equidepressor both before and after injection of atropine into the cat, indicating, as would be expected, that choline passes to the cathode like histamine.

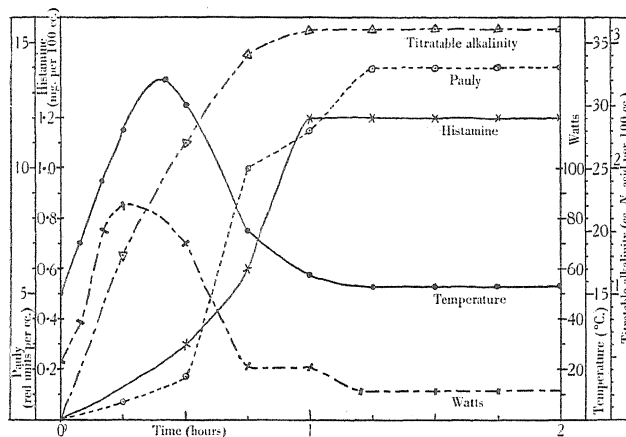


Fig. 1. Electro dialysis of 20 g. lung in 100 cc. cell.

- × — — × Histamine values of cathode liquid.
 o — — o Pauly values of cathode liquid.
 Δ — — Δ Titratable alkalinity values of cathode liquid.
 ● — — ● Temperature of mixture in centre compartment.
 + — — + Watt values.

Reliability of end-point. In nine experiments samples were withdrawn at 15 minute intervals, neutralised and injected into an anaesthetised cat. The results from one of these experiments are shown in Fig. 1. Similar curves were obtained in the other experiments. They showed that:

1. The Pauly value never attained a maximum before the histamine concentration.
2. The titratable alkalinity value usually reached a maximum before the histamine concentration.
3. The current usually but not always reached a minimum after the histamine concentration became maximum.

Thus the Pauly reaction was the only reliable method of the three for determining the end-point.

Recovery of added histamine.

1. *From histamine-free material.* A known volume of histamine solution was added to a 30 % blood solution and electro dialysed. The same volume of histamine was added to an appropriate volume of water. The dialysate was then

concentrated *in vacuo* to this volume, and the two solutions were sterilised and preserved until compared on the cat's blood pressure. The amounts of histamine added were such as to give a similar concentration in the cell to that given when a tissue was being dialysed, *i.e.* 2, 1 or 0.5 mg. were added to the 250 cc. cell and 0.8, 0.4 or 0.2 mg. to the 100 cc. cell. These amounts correspond to tissues containing 40, 20 and 10 mg./kg. respectively. In nine experiments the base was recovered quantitatively so far as could be detected by the physiological assay. It has already been stated in a preliminary communication [MacGregor and Thorpe, 1933] that a large amount of histamine (0.06 g.) added to blood was easily recovered in the form of its picrate in good yield.

2. *From tissues containing histamine.* Failure to recover completely histamine which has been added to tissues has often been reported. These experiments have been carried out by adding histamine to one portion of the tissue and working up another portion without added histamine. The difference between the two extracts is then estimated by matching them against a standard histamine solution on the cat's blood pressure, and hence the amount of added histamine is calculated. The method, especially when the differences are great, is liable to big errors owing to the large multiples involved in the calculations. In our experience the stronger solutions tend to be underestimated. Being of the opinion that this was largely responsible for the failures to recover added histamine, we planned our experiments so as to use equipotent solutions and to eliminate all calculations.

A known volume of a histamine solution was added to a portion of the tissue and the mixture dialysed. An equal portion of the same tissue was dialysed in another cell and the same volume of histamine added to the dialysate after removal from the cell. The two dialysates were then neutralised, adjusted to the same volumes and equal doses compared on the cat's blood pressure. In five experiments no difference could be detected between the two solutions. The histamine had been recovered quantitatively within the limits of the physiological assay.

SUMMARY.

1. A rapid method for the quantitative extraction of histamine from tissues by electro dialysis is described.
2. The method gives results in complete agreement with the alcohol method.
3. Added histamine is recovered quantitatively within the limits of the physiological assay.

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CLXXXIX. A STUDY OF THE COMPOSITION OF HUMAN MILK.

THE INFLUENCE OF THE METHOD OF EXTRACTION ON THE FAT PERCENTAGE.

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(Received July 15th, 1933.)

THE fat content of both cow's and human milk is a subject on which much work has been done, and though certain facts are now well established there is still no clear understanding of the circumstances which determine the normal range of variation or knowledge of the conditions which determine these comparatively wide limits. In the course of our study on the composition of human milk in the early stages of lactation [Lowenfeld *et al.*, 1927; 1928] it was noticed that the method of extraction of the milk sample had a considerable influence on the percentage of fat, although no other constituent was affected by this factor. Since this factor had not to our knowledge been investigated by any previous worker, it occurred to us that herein might lie the probable explanation of the variations in the fat percentage hitherto recorded. We decided therefore to undertake a more intensive study of the fat content in human milk throughout the whole course of lactation, paying special attention to the method adopted for obtaining the milk sample. The present paper is a record of our results and the conclusions which they suggest.

METHODS OF OBTAINING MILK.

The two methods used for obtaining the samples of milk (for convenience called the "extraction" of milk) throughout the work were either digital expression or gentle use of the breast pump, and in every case where milk dripped from the breast a sample of this milk was also collected in addition to the other samples.

These two methods were adopted after critical observation of the actual process of suckling by an infant. This process consists of two factors, the direct pressure exerted by the gums of the baby upon the nipple and the areola of the breast, and the suction action exercised by tongue and cheek drawing on the openings at the end of the nipple. Digital pressure and gentle suction from a pump used without pressure upon the breast represent the nearest approach which can be made artificially to reproduction of these two factors in the normal process. Systematic comparison made of the percentages of fat in samples of milk extracted by these two methods, under similar conditions, at once showed very marked differences. Indeed, the differences were so striking that we have come to the conclusion that in the method of extraction is to be found the chief explanation of the wide variations shown in fat figures obtained by different workers.

MATERIAL.

Twenty hospital cases were used for investigation of the milk of the first fortnight of lactation, and a continuous study of the milk of the same mother throughout this period was thus possible.

Owing to irregularities in attendance at hospital or clinic, it was not possible to achieve the same continuity in the later period of lactation. Nevertheless, every effort was made to examine the milk of the same woman over as many months as possible. By a combination of the figures obtained from the two sets of investigations, material sufficient for a general survey of the problem of the fat percentage in milk under varying conditions and during the whole course of lactation has been obtained.

The milk of 51 mothers was examined for the percentage of fat, protein, sugar, calcium, phosphorus and ash, and the results of this work, except for that on fat, have already been recorded [Lowenfeld *et al.*, 1927; Widdows *et al.*, 1930]. The milk samples were obtained from nursing mothers in the lying-in wards of the Obstetric Unit of the Royal Free Hospital, from those attending the Shore-ditch Carnegie Welfare Settlement, and the Mothercraft Training Society, Cromwell House, Highgate.

Careful notes were taken of the mother and child so that all abnormal factors could be excluded. In every instance, except for ten cases, the baby was thriving satisfactorily. In these ten cases an attempt was made to determine the cause of the nutritional disturbance in the infants.

To maintain uniformity of conditions the milk was always taken for analysis at the same feeding time, and the same interval was observed between the times of feeding. The importance of these factors had been shown in the case of cows by Eckles and Shaw [1913] who state that the percentage of fat is usually highest about midday, and in a Bulletin recently issued by the Ministry of Agriculture and Fisheries [1931] instances are given from the work of Gilchrist [1925] and Mackintosh [1925] fully confirming this diurnal variation. In the case of human milk, Helbich [1911] showed the highest percentage to be at 10 a.m. or 2 p.m., and more recently Deem [1931] has again emphasised this, showing that the percentage of fat is at a maximum at 10 a.m., nearly the same at 2 p.m., and at a minimum at 6 a.m.

In our work all the samples in the early days of lactation were taken at 10.30 a.m. and during the later periods at 2 p.m.

The taking of the sample, except in the case of the Mothercraft Training Society, was carried out by one of us (M. F. L.) and the biochemical analysis by the other (S. T. W.).

Every visit of each mother necessitated, as will appear later, at least four estimations of fat, and where milk dripped from the breast a sample of this dripped milk was also taken for estimation. In all, 276 samples taken before and after the baby had fed and 18 dripped samples were collected and analysed.

ANALYTICAL METHODS.

Gottlieb's method was used for the estimation of the fat.

To study the influence of the method of extraction on the fat content of the milk 216 samples were examined. These were obtained according to the following procedure.

From one breast a sample sufficient for analysis was taken by hand, the amount varying from 2 to 30 cc. according to the average yield of the subject.

The baby was then allowed to feed from 2 to 5 minutes, and the breast was then finally emptied by hand, the total resulting fluid being considered as the second sample.

From the other breast also a sample was taken before feeding, by hand; the baby was allowed to feed for the same length of time as before, and the final sample, which emptied the breast, was obtained by gentle use of the pump, avoiding pressure upon the breast.

The use of the two different methods, keeping the other conditions constant, enabled the bearing of each upon the fat percentage to be studied. On the one side a uniform method of extraction was employed, namely digital expression, for both the samples. On the other side digital expression was used in obtaining the first sample and suction by the breast pump for the last sample.

Our previous work has shown that in normal cases the composition of the milk given by either breast is approximately the same. Assuming this to be generally the case in all women, if the manner of extraction were immaterial, even although the exact figures of the percentage might differ on either side, a rise in the fat percentage on one side would be paralleled by a similar degree of rise on the other. It is therefore the question of the relation of the method of extraction to the production of the degree of rise in the fat percentage between the beginning and end of a feed with which we are at present concerned.

Before considering our results, certain factors that occur during breast feeding must be taken into consideration. Stimulation of one breast produces immediate changes in the other breast, and in certain women the breast of one side will begin to leak as soon as extraction starts from the other; loss of fore-milk thus occurs from the second side. Furthermore, the amount taken by the baby on each side is impossible of exact regulation. Thus, in spite of all care, the two samples taken from either side cannot be exactly equivalent. Apart from these discrepancies, which were beyond our control, all other conditions were kept uniform for the two sides. A study of the figures obtained shows that in very few cases is there a general agreement in the degree of rise of fat on the two sides. Indeed it is apparent that the change of method from hand to pump for the last sample of the second side materially affects the normal rise in the second sample of the side under consideration.

EXPERIMENTAL RESULTS AND THEIR ANALYSIS (Tables I, II and III).

In Table I (A, B and C) are given 25 illustrative instances representing 22 women, where the effect of extracting the samples by suction with a minimum degree of pressure upon the breast was either to lessen the expected rise in fat percentage of the second sample or actually to obliterate it.

In Table I A it will be seen that there is an increase in the percentage of fat in the samples taken after the feed, whether extracted by hand or pump. This is in accordance with the findings of previous workers both in the case of cows' milk [see Eckles and Shaw, 1913; Jensen, 1906; Wellmann, 1911; Min. Agric. Fish., 1931], and human milk [see Adriance, 1897; Söldner, 1896; Schlossmann, 1900; 1902; Myers, 1927]. We ourselves have found with regard to the individual feed that in every case in which a single method of extraction was used throughout the taking of the milk, whether it were breast pump, digital pressure, or a mechanical milker¹, the later portion of the milk was richer in fat than the earlier. It is now an accepted fact that the strippings in any milk extraction show a higher percentage of fat than the middle or early milk of the same extraction.

¹ Dr Chisholm very kindly tested this method for us in Manchester.

Table I.

(The following abbreviations are used in the tables: L, left; R, right; B, before feed; A, after feed; H, hand extraction; P, pump extraction; m, month; w, week.)

Case (age of baby below)	Breast	Before feed			A	After feed			Rise in % of fat	Reduction of expected % increase (after pump extraction)
		Volume in cc.	Method of ex- traction	% of fat		Volume in cc.	Method of ex- traction	% of fat		
1. (H 2)	R	7	H	5.00	A	15	H	8.88	3.88	2.42
	L	7	H	3.79		13	P	5.25	1.46	
2. (R)	L	4.5	H	3.12	A	3.3	H	9.18	6.06	3.34
9 m	R	4.5	H	4.69		2.8	P	7.41	2.72	
3. (S)	R	22	H	0.73	A	36	H	4.19	3.46	1.54
7 m	L	15	H	3.00		14	P	4.92	1.92	
4. (T)	R	11	H	1.09	A	12	H	6.16	5.07	1.83
5 w	L	9.5	H	4.52		11	P	7.76	3.24	
5. (Ga)	R	18	H	3.06	A	23	H	5.69	2.63	1.30
3 w	L	11	H	4.22		7	P	5.55	1.33	
*6. (Co)	R	11	H	3.74	A	11	H	11.13	7.39	4.11
5 m 3 w	R	14	H	2.06		5	P	5.34	3.28	
7. (S. i)	R	9	H	3.97	B	10	H	8.73	4.76	0.64
	L	13	H	5.58		1.8	P	9.70	4.12	
8. (S. ii)	L	7	H	4.97	B	15	H	6.42	1.45	0.08
	R	28	H	4.13		1.5	P	5.50	1.37	
9. (J. i)	R	10	H	3.62	B	5.5	H	8.40	4.78	2.63
2 m	L	22	H	2.60		1	P	4.85	2.25	
10. (J. ii)	R	7.5	H	2.65	B	7.5	H	5.21	2.56	1.52
2 m 3 w	L	2.3	H	2.60		1.4	P	3.64	1.04	
11. (N. R.)	R	11	H	5.07	B	22	H	8.29	3.22	1.05
3 m	L	14	H	5.64		1.7	P	7.81	2.17	
12. (Y)	R	11	H	4.61	B	22	H	6.74	2.13	1.02
5 w	L	13	H	4.09		5.5	P	5.20	1.11	
13. (R. R.)	L	6.2	H	4.12	B	12	H	6.92	2.80	1.21
3 m	R	15	H	4.32		5.5	P	5.91	1.59	
14. (B)	R	8	H	1.26	B	20	H	4.93	3.67	1.20
	L	5.5	H	3.21		2.8	P	5.68	2.47	
15. (Cp)	R	6	H	2.40	B	20	H	5.00	2.60	1.24
3 m 3 w	L	11	H	4.08		5	P	5.44	1.36	
16. (Po)	R	9.5	H	1.28	B	9	H	4.60	3.32	2.47
7½ m	L	6	H	4.48		2.5	P	5.33	0.85	
17. (Sh)	L	10	H	2.29	B	8.5	H	7.00	4.71	3.28
	R	7	H	5.73		2	P	7.16	1.43	
18. (Ha)	R	16	H	4.73	C	3	H	6.60	1.87	1.85
3 m	L	6	H	6.67		3.5	P	6.69	0.02	
19. (Bl)	R	4	H	1.71	C	7.5	H	3.59	1.88	1.37
	L	3.5	H	2.73		3.5	P	3.24	0.51	
20. (Kn)	L	5	H	4.88	C	11	H	8.76	3.88	3.40
5 w	R	11	H	7.62		1	P	8.10	0.48	
21. (H. i.)	R	7	H	3.07	C	15	H	5.32	2.25	1.95
	L	7	H	2.88		13	P	3.18	0.30	
22. (Co)	L	5	H	3.54	C	6.5	H	6.30	2.76	2.72
9 w	R	4	H	2.12		2.5	P	2.16	0.04	
23. (Si)	L	7.3	H	1.66	C	26	H	4.70	3.04	2.59
2 m 2 w	R	6	H	6.49		10	P	6.94	0.45	
24. (G)	L	5.5	H	2.24	C	15	H	4.49	2.25	1.98
7 w	R	4	H	3.00		7.5	P	3.27	0.27	
25. (Ra)	R	13	H	2.01	C	9	H	4.91	2.90	2.60
3 m	L	12	H	5.20		3.5	P	5.50	0.30	

* As the patient had only one breast, the figures were taken on two different occasions, very close together, from the remaining breast.

Volumes. In cases 1, 2 and 4, the volumes of the samples are approximately equal. In 5 and 6 the question of the volumes falls into a grouping to be considered later. In each case the sample taken by pump contains less of the middle milk than that taken by hand.

On careful examination, however, the extent of the rise in fat will be found to be much less on the side where the final extraction was made by pump than that on the side taken by hand.

Table I B shows 11 cases where the volume obtained in the sample extracted by pump in the after-feed is very much smaller than that of the sample obtained by hand, indeed, in many cases the pump sample may be considered to consist of strippings only. In spite of this fact, it will be found that the rise to be expected in the after-sample, and shown definitely in that taken by hand, is reduced. To this table should be added cases 5 and 6 (Table I A) already referred to.

In the case of the 8 women whose samples of milk are shown in Table I C, the fat percentage of the sample taken by pump after the feed, when compared with that of the sample taken by hand before the feed, shows so small a rise (0.02 to 0.51 %) as to represent, within the limits of experimental error, practically stationary values; although on the other side at the same feed, the samples taken by hand show a consistent rise from 1.87 to 3.88 % over that taken by the same method before the feed on that side.

In certain women the process was carried even further, as will be seen in the 7 cases in Table II. Here, although on the side where hand extraction was used for both the fore- and after-samples, the normal and expected rise in the fat

Table II.

Case	Breast	Before feed			After feed			Rise or fall in % of fat
		Volume in cc.	Method of extraction	% of fat	Volume in cc.	Method of extraction	% of fat	
26. (La)	R	5.3	H	1.86	12	H	4.23	2.37
	L	6.5	H	3.16	5.5	P	3.13	-0.03
27. (Va)	R	8	H	5.83	6.5	H	9.87	4.04
	L	7	H	4.91	3.5	P	4.40	-0.51
28. (O. F.)*	L	7.5	H	1.78	36	H	5.28	3.50
	R	12	H	7.264	4	P	7.12	-0.14
29. (Fo)	L	10	H	4.81	7.5	H	9.80	4.99
	R	15	H	4.16	15	P	2.13	-2.03
30. (Sm)	L	4.5	H	1.19	6	H	4.36	3.17
	R	3	H	3.27	5.5	P	2.14	-1.13
31. (Ba)	L	4.5	H	0.64	11	H	2.33	1.69
	R	5	H	3.16	2.5	P	2.96	-0.20
32. (Wi)*	L	9.7	H	2.85	11	H	4.02	1.17
	R	9.5	H	6.26	11	P	5.89	-0.37

* In cases 28 and 32 there was a very marked difference in % of fat in "before feed" samples in the two breasts, probably due to feeding habitually from one breast only.

percentage of the after-sample occurred, the rise ranging from 1.17 to 4.99 %; on the other side where hand and pump were used for extraction, not only did no rise occur, but the percentage of fat in the after specimen was actually lower than that in the one taken before the feed.

As is usual in dealing with the human organism, the expected does not invariably occur, and we have had a few cases in which extraction by pump has not affected the percentage of fat in the sample taken after the feed in the manner to be expected. In Table III are given five such cases.

Table III.

Case	Breast	Before feed			After feed			Rise in % of fat
		Volume in cc.	Method of ex- traction	% of fat	Volume in cc.	Method of ex- traction	% of fat	
33. (Hy)	R	8	H	3.59	17	H	5.02	1.43
	L	12	H	2.62	5.5	P	4.09	1.47
34. (So)	L	7.5	H	2.69	15	H	3.62	0.93
	R	4	H	3.30	8	P	4.34	1.04
35. (Gr)	R	2.5	H	3.70	9	H	6.99	3.29
	L	10	H	3.32	6	P	6.68	3.36
36. (Ho)	R	15	H	2.50	13	H	4.17	1.67
	L	7.5	H	2.70	7.5	P	5.00	2.30
37. (Yt)	R	6	H	4.70	20	H	5.09	0.39
	L	10	H	2.42	14	P	4.28	1.86

Although the influence of the method of extraction upon the percentage of fat was shown unmistakably in all but the above cases, yet it is discernible even in these. In all except 35, it will be noted that the volumes after extraction by pump are considerably smaller than those taken by hand. In view of this fact, it would be expected that the normal rise shown by any method of extraction would be greater between a sample taken before feeding, of volume 12 cc., and a sample taken after feeding of volume 5.5 cc., than in a sample of 8 cc., before and a sample of 17 cc. after feeding. Nevertheless the actual rise in the former case is practically the same as in the latter. Indeed in cases 33, 34 and 35 the rise on both sides, within the limits of experimental error, is equal. In cases 36 and 37 we present the only cases found in our total series in which the percentage of fat in the after-sample taken by pump is significantly greater than in the sample taken by hand. It will be noted that the volumes of the after-samples in extraction by pump are considerably smaller in both cases than those taken by hand.

DEDUCTION FROM ABOVE EXPERIMENTAL DATA.

As has been pointed out already, the normal action of a baby feeding upon a breast can be analysed into two factors: pressure and suction; and it would appear from a study of the preceding tables that the pressure is the important factor in the production of a high percentage of fat. The greater the pressure apparently the steeper will be the rise in the fat percentage. Suction employed alone, on the other hand, tends to lower the percentage of fat. The larger, therefore, the element of suction in the act of extraction, the smaller apparently will be the ultimate rise in the fat percentage.

As will be indicated later, there appears to be an inverse ratio between the volume of milk in the breast at any time during any one feed and the percentage of fat in the sample. The larger the quantity of milk at any moment, the lower the percentage of fat in that milk—the smaller the quantity of milk, the higher the fat content. In the experiments just described, these factors have been arranged so that they act in opposition to each other.

It is suggested that the following conclusion may be formulated from these data.

In any given sample of milk, other factors being equal, the percentage of fat present will depend inversely upon the quantity of milk present in the breast at the time of taking the sample, and directly upon the degree of pressure exerted upon the areola and nipple in the process of extraction.

If this be true, then milk which has dripped from a breast, that is to say, which has been extruded in answer to conditions within the gland and not by any means of extraction from without, should theoretically give the lowest percentage of fat of all, and this is exactly what occurs.

EXPERIMENTAL RESULTS ON DRIPPED MILK.

Table IV shows the relation of the percentage of fat in milk which has dripped from a breast to that extracted by other means. In every case it will be seen that the milk which drips is lower in its fat percentage than that obtained by extraction, whether by pump or hand.

Table IV.

b. f., before feeding; a. f., after feeding; D, dripped.

Case	Breast	Method of extraction	% of fat	Method of extraction	% of fat	Rise in % of fat
38. (Je)	R	D (b. f.)	0.26	H (b. f.)	2.94	2.68
39. (Kn)	L	D "	2.77	H "	4.88	2.11
40. (Co)	L	D "	1.57	H "	5.66	4.09
41. (Wi)	L	D "	1.78	H "	3.92	2.14
42. (Te)	R	D "	0.35	H "	1.09	0.64
43. (Pe)	R	D "	2.24	H "	5.52	3.28
44. (Wa)	R	D "	1.82	H "	2.36	0.54
45. (Ha)	R	D (a. f.)	1.12	H (a. f.)	4.02	2.90
46. (Gr)	R	D "	2.54	H "	5.75	3.21
47. (Gi)	L	D "	1.65	H "	4.49	2.84
48. (Gr)	L	D "	3.64	P "	6.16	2.52
49. (Sm)	L	D "	2.30	H "	4.36	2.06
50. (Hv)	R	D "	2.30	H "	5.02	2.72

As will be seen from Table IV, the first 7 cases show the percentage of fat in milk which has dripped to be lower than in milk taken by hand, before feeding took place or any other milk was extracted. In the last 6 cases the stimulation of suckling the baby at one breast caused the other breast to drip. These dripped samples so obtained are compared with samples previously taken from that same breast by hand or pump. It is of interest that in cases 47 and 50 milk taken by hand before feeding gave 2.24 and 3.59 % of fat, that is, 0.59 and 1.29 % more than the milk which dripped after feeding. In all other cases the fat percentage in the dripped milk was less than that in the specimen taken immediately after the feed either by hand or by pump.

It may therefore be concluded that when samples are taken from any given breast, all conditions other than the method of extraction being similar, a specimen of milk which has dripped from a breast will show the lowest proportion of fat obtainable at that moment. Nevertheless, the figure in itself need not actually be low, since the woman under examination may be one of those women who, under all circumstances, produce a high level of fat. This was shown in a case (Ra) in which the percentage of fat in the dripped milk before feeding was 4.39 rising to 6.26 and 8.63 in the before- and after-specimens taken by hand; or another case (R. R.) in which the dripped sample before feeding showed a percentage of 3.18 rising to 4.69 in the hand sample before feeding and 7.41 in the pump after-sample.

Adjustment to the method of extraction, though it occurs eventually, is of course never immediate. If a very small sample be taken by hand immediately after the dripped sample, the rise in the fat percentage, though definite, will in all probability be slight. As for instance in the case (So) when the percentage in

the sample dripped before feeding was 4.86 rising to 4.96 in the sample taken by hand before, and 6.42 in the sample taken by hand after, feeding; and another case (Pa), where the dripped sample before feeding showed a percentage of 3.15 rising to 3.55 and 5.33 in the samples taken by hand before and after feeding.

OTHER FACTORS INFLUENCING THE PERCENTAGE OF FAT IN THE MILK SAMPLE.

There are certain other factors which have an influence on the percentage of fat in the sample of milk extracted, when the same method of extraction (either digital pressure or breast-pump) is employed.

These factors have been taken into account when considering the effect of the mechanism of the extraction upon the composition of the sample.

1. *Period of lactation.* It has been found that the proportion of fat in the milk of all multiparae investigated rises between the first and fourteenth days. The nature of the rise is shown in Table V.

Table V.

Case	Day of lactation	% of fat	Case	Day of lactation	% of fat
51 (J)	2	1.40	53 (H)	6	0.44
"	3	1.90	"	7	3.10
"	4	2.57	"	8	3.35
"	5	2.82	"	9	3.80
"	9	4.50	"	10	4.60
52 (P)	5	3.26	54 (Pd)	2	2.02
"	6	4.20	"	5	3.40
"	7	3.73	"	6	3.42
"	8	3.49	"	7	3.88
"	9	4.15	"	8	4.15
"	10	5.43	"	12	4.58
			"	13	4.95
			"	14	4.72

In the case of primiparae it is found that the percentage of fat in the early part of the feed is also as a rule higher than at the end, but, as shown in Table VI, this is not invariable.

Table VI.

Early milk of primiparae					
Case	Day	Breast	% of fat in sample		
			Before feed	After feed	Difference
55. (F) primipara	1st	R	0.95	0.53	- 0.42
" "	2nd	L	1.66	1.26	- 0.40
" "	3rd	L	2.73	3.35	+ 0.62
56. (P) "	2nd	R and L	3.13	2.72	- 0.41
" "	4th	L	3.04	4.56	+ 1.52
" "	5th	R	1.92	4.16	+ 2.24
" "	14th	L	3.55	5.89	+ 2.34
57. (Pd) "	2nd	R	7.14	5.02	- 2.12
(Small "	2nd	L	6.92	5.74	- 1.18
quantities)	6th	R	5.44	5.60	+ 0.16
" "	6th	L	9.98	8.66	- 1.32
" "	7th	R	4.36	6.10	+ 1.74
" "	7th	L	3.64	9.02	+ 5.38
58. (J) multipara	3rd	R	1.57	2.12	+ 0.55
" "	4th	L	2.57	3.09	+ 0.52
59. (Bk) "	2nd	L and R	3.50	6.05	+ 2.55
" "	3rd	R	2.97	4.09	+ 1.12

2. *Individuality of women.* In the case of the milk of women examined during the first fortnight of lactation, it was found that although the mean percentage of fat in all cases rose from the first to the fourteenth day, yet in some cases the highest figure shown was between 3 and 4 %, although in other cases the percentage rose quickly to over 4 % and then to 5 % and over 6 %.

In the later periods of lactation, although two different methods of extraction were being used, the same general phenomenon was noted. In some women the lowest value for the fat obtained was rarely less than 4 % (usually between 5 and 6 %), and in some women over 7 %. In others the lowest value was less than 3 %, falling generally between 3 and 4 % and rarely rising above 4 %.

These observations suggest that some women throughout their whole lactation period will have normally a much higher percentage of fat in their milk than others. This individual variation has been observed also in the case of cows [see Min. Agric. Fish., 1931].

3. *Volume in the breast.* Emphasis has been laid in the foregoing work on the quantity of fluid obtained at any one extraction, and it might have been expected that a difference in total quantity habitually secreted would be reflected in the fat content of the milk.

Engel and Frehn [1910] consider that there is such a relationship, and that the milk output and the fat content are inversely related to one another. From a study of our own cases, there seems no such clear relationship. In Table VII are

Table VII.

Case	Age of baby	Sample	% of fat
60. (P)	First two weeks of life	2	6.17
"	"	3	4.22
"	"	4	4.50
"	"	7	7.40
"	"	8	5.82
"	"	9	5.20
61. (F)	"	1	0.74
"	"	2	1.45
"	"	3	3.00
"	"	7	2.44

given the average percentages of fat obtained from samples from two different women on successive days, and although in each the quantity of milk was less than 10 cc. a wide difference is shown in the percentages of the fat.

If the usual quantity of fluid secreted by the breast of an individual suddenly changes, that is to say, if in a mother who habitually secretes a large amount of milk the quantity quickly decreases, or *vice versa*, then we have found that this change will affect the fat and be reflected in a rise or fall respectively. Thus, in one case when the fat percentages on the 8th and 9th days after parturition were 4.92 and 5.03 respectively, the volume of milk in the breast was small in consequence of extreme heat and profuse sweating. On the 10th day, however, after the intake of a great deal of fluid, the volume of milk in the breast suddenly rose and the percentage of fat fell to 3.8 %. The figures in Table VIII further illustrate this point.

Therefore it would appear that the final percentage of fat occurring in the milk at any given period during a feed is the product of several factors—the nature of the habitual yield of the individual woman, the place in the feed, and the mechanics of the process of extraction.

Table VIII.

Case	Volume in cc.	% of fat
62. (F)	3.3	2.73
"	5.5	3.35
"	20	1.52
63. (P)	Quantity under 10	6.17
"	"	4.22
"	"	4.50
"	"	7.40
"	"	5.82
"	"	5.20
"	38	2.98

The above work, which was in the first instance undertaken to elucidate the variations in the fat content of milk recorded by many other workers in this field, has proved itself of value from the clinical point of view. This side of the work will be recorded in a separate communication.

CONCLUSIONS.

From our results it is suggested that the following conclusions may be drawn.

1. Differences in the method of extraction of a sample of milk appear to account for the wide variations in the fat figures hitherto obtained by different workers.

2. In any given sample of milk, other factors being equal, it would appear that the percentage of fat present will depend inversely upon the quantity of milk present in the breast at the time of taking the sample, and directly upon the degree of pressure exerted upon the areola and nipple in the process of extraction. The lowest percentage of fat will be found in milk which has dripped spontaneously from the breast.

3. There appears to be an individual variation in the average fat content of the milk of different women.

4. There would seem to be no clear relationship between a permanently high or low milk yield and the proportion of fat in the yield, though this proportion appears to be affected by sudden variations in the yield.

5. In all women investigated, the percentage of fat in the milk examined showed a rise between the first and the fourteenth days.

We wish to express our thanks to Professor Dame Louise MacIlroy for permission to carry out parts of this study in the wards of the Obstetric Unit of the Royal Free Hospital; to Dr Chodak Gregory for facilities for observation in the Infant Welfare Department of the Royal Free Hospital; to Miss Liddiard and sisters of the Mothercraft Training Society for valuable assistance; and to Dr Olivier Richards and the staff of the Carnegie Model Welfare Centre for co-operation in the selection of patients. We also wish to thank Professor Winifred Cullis and Miss M. Bond for much valuable criticism and advice; and Dr Shiskin for help in searching the literature. Finally we acknowledge with gratitude help received from the Medical Research Council and the Waller Research Fund which has made this work possible.

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CXC. THE EFFECT OF ULTRA-VIOLET, RADIUM AND X-RAY RADIATION ON GLUTATHIONE IN PURE SOLUTION.

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(Received July 19th, 1933.)

GLUTATHIONE has recently been shown to influence the activity of many enzyme systems [Pringsheim *et al.*, 1931; Lohmann, 1932; Waldschmidt-Leitz *et al.*, 1933] and has received considerable attention as a possible regulator of growth in the cancer cell. In view of the retarding action of radium and X-rays on cancerous growth and of the lethal effect of ultra-violet radiation of shorter wave-length than 2900 Å., the question arises as to the effect of these rays on the glutathione molecule. Accordingly the effect of such radiation on pure glutathione in aqueous or buffered solution has been investigated with regard to the fate of the sulphur in the molecule.

ULTRA-VIOLET RADIATION.

Glutathione solutions were exposed to radiation from the full quartz mercury arc¹ at a distance of 25 cm. The solutions were contained in flat dishes, giving thin layers of solution, and covered with transparent quartz plates. The dishes were surrounded by an ice-bath during the radiation to remove any possibility of destruction by heat. Samples were removed at intervals, made to 2 % sulphosalicylic acid and titrated by the iodate procedure [Woodward and Fry, 1932] for the reduced glutathione remaining. The total glutathione remaining was also determined after zinc reduction in the acid solution. By analogy with known sulphur compounds, glutathione which has been oxidised as far as its disulphoxide form (G.SO.SO.G) would be included in this figure. Table I shows the results obtained.

Table I. *Decomposition of glutathione by ultra-violet radiation.*

<i>p</i> _H	Original glutathione concentration mg. per 100 cc.	Glutathione remaining after radiation					
		4 hours		6 hours		8 hours	
		Reduced %	Total %	Reduced %	Total %	Reduced %	Total %
3.5	50	51	75	—	—	—	—
3.8	33	50	77	—	—	—	—
7.0	33	16	63	6	—	4	40
7.3	33	0	35	—	—	—	—
6.8	200	35	62	—	—	—	—
7.5	200	14	44	12	44	—	—
8.0	200	13	52	9	48	—	—

¹ A Uviarc Laboratory Arc, 3.5-4.5 amperes, 110 volts D.C.

The solution radiated at p_H 3.5 was unbuffered, being at the p_H of pure aqueous glutathione. The other solutions were buffered in 0.02 *M* phosphate. It will be observed that there was a very rapid loss in total glutathione, that is, it was changed in such a way that it could not be reduced back to a sulphhydryl compound by nascent hydrogen. Glutathione not determined by this analysis will be considered "destroyed" since it can no longer act as a member of the biologically active oxidation-reduction system, $2G.SH \rightleftharpoons G.S.S.G.$

The amount of destruction of glutathione as well as oxidation of sulphhydryl is shown to be dependent upon the concentration, p_H and time of radiation. In the higher concentrations, a smaller percentage loss of glutathione was observed. The effect of p_H is shown in Table I. At a concentration of 33 mg. per 100 cc., slight changes in p_H around 7.0 produced much greater increases in both the oxidative and destructive effects than at more acid p_H values. Increasing the p_H from 7.5 to 8.0, however, in the higher concentration did not cause any greater effect. At constant p_H (7.0) oxidation of sulphhydryl to disulphide glutathione proceeded much more rapidly than destruction and is, therefore, probably a primary step in the destructive effect of the radiation.

Using the closely related sulphur compounds, cysteine and cystine, Lieben and Molnar [1931] have shown a similar destructive effect of ultra-violet light.

The fate of the sulphur in the glutathione which was destroyed was investigated in the solutions of higher concentration. Free sulphate could be detected in all these by means of barium sulphate precipitation. Analytical fractionation of the forms of sulphur present was therefore made by a procedure adapted from the macro-method of Denis and Reed [1927] for sulphur compounds in blood.

Total sulphur and inorganic sulphide. No sulphur was lost as gaseous products, since 100 % was recovered as barium sulphate after oxidation by Benedict's [1909] copper nitrate oxidising reagent. Therefore no inorganic sulphide was formed. This was confirmed with lead acetate paper after acidifying and boiling the solution.

Sulphates. Both free and hydrolysable sulphate were found. There was some barium sulphate precipitation even if the solution was not boiled with acid, representing the free sulphate in the solution. This was removed after standing 20 hours, and a further precipitate of barium sulphate was obtained on gentle boiling. This was classified as the hydrolysable sulphate and is probably of the sulphonate ester type.

Intermediate sulphur. This classification includes the sulphur which was not determined in the analyses for total glutathione and total sulphate. It was

Table II. *State of sulphur after ultra-violet radiation of glutathione solutions.*

Type of sulphur compound	p_H and time of radiation		
	p_H 6.8, 4 hours	p_H 7.5, 5 hours	p_H 8.0, 6 hours
Total sulphur recovered	101.0	101.5	99.5
Total glutathione { GSH			
GSSG	62	44	48
Sulphoxide			
Free sulphate	8.4		16.4
Hydrolysable sulphate (sulphonate)	4.2		5.5
Total sulphate	12.6	21.6	21.9
Inorganic sulphide	0	0	0
Intermediate sulphur { Sulphone			
R_2S	25.4	34.4	30.1

calculated by subtracting the sum of these two determinations from 100 %, and probably includes sulphur of the organic sulphide (R_2S) and sulphone ($R-SO_2-SO_2-R$) types. No free sulphur could be detected, although all solutions were slightly yellowish after radiation.

Table II summarises the analytical results thus obtained. The destruction produced seems to be due to a progressive oxidising effect of the ultra-violet radiation rather than to liberation of inorganic sulphide or free sulphur from the molecule.

RADIUM.

Radon in both glass and gold seeds was used in studying its effect on glutathione. The seeds were placed directly in 10 cc. of glutathione solution, in the first case (Table III), at p_H 3.5, unbuffered and containing 50 mg. per 100 cc.; at the higher p_H values in 0.02 *M* phosphate buffer containing 33 mg. per 100 cc. The flasks were stoppered and left to stand at room temperature. Control solutions without radium were placed so as to receive no radiation from the experimental solutions (Table III).

Table III. *Effect of radium on pure glutathione.*

p_H	Duration of treatment hours	Glass seeds ($\beta + \gamma$ rays)				Gold seeds (γ rays only)			
		Amount of radiation m.c.h.	Glutathione present after treatment		Amount of radiation m.c.h.	Glutathione present after treatment		Control Glutathione remaining	
			Reduced %	Total %		Reduced %	Total %	Reduced %	Total %
3.5	25	212	75	97	—	—	—	87	100
	68	469	31	86	—	—	—	78	100
7.0	4	38	77	99	34	79	99	88	100
	24	213	0	84	191	2	92	4	100
7.3	4	32	67	95	29	70	95	65	99
	25	180	0	85	160	0	86	0	99

The results show that glutathione is extremely resistant to both the β - and γ -rays of radium. At the acid p_H , after 212 millicurie hours of treatment, there was some oxidation, but only very slight destruction. After longer treatment, 469 millicurie hours, both effects were more pronounced. At the more alkaline p_H values the autooxidation of glutathione was so rapid that the oxidative effect of the rays was not as definite. However, the destructive effect was still quite evident.

The oxidative effect observed by Hammett [1932] as expressed by a decreased nitroprusside test is thus quantitatively demonstrated.

X-RAYS.

Glutathione in solutions similar to those used for the radium treatment was exposed for 4 hours to unfiltered X-rays at 45 cm. distance from a machine running at 120 kv. The glutathione solution was contained in a stoppered pyrex glass flask and surrounded by ice-water for cooling during the radiation. At the more alkaline p_H values some autooxidation naturally occurs during the course of 4 hours. To allow for this a control solution was allowed to stand in ice-water for the same length of time. The results (Table IV) showed that there was no effect on the glutathione which could be attributed to X-rays. The

Table IV. *Effect of X-rays on pure glutathione.*

p_{H}	Duration of treatment hours	Amount of radiation R units	Radiated solution			
			Glutathione present after radiation		Control Glutathione present	
			Reduced %	Total %	Reduced %	Total %
3.5	4	15,600	95	99	100	100
3.8	4	15,600	93	104	100	100
7.0	4½	10,800	80	103	72	100
7.3	4	15,600	56	102	65	99

stability of compounds of this type toward X-rays has also been reported by Stenström and Lohmann [1928] who found no destruction of cystine in acid solution when exposed for as long as 95 hours.

SUMMARY.

Ultra-violet radiation was found to produce a very destructive effect on glutathione, radium a slight destructive effect, both of these agents causing at the same time partial oxidation of the sulphydryl to the disulphide form. X-rays had no effect on the glutathione.

The effect of ultra-violet radiation appeared to be of the nature of an oxidative destruction. Free sulphate was formed to the extent of 42 % of the glutathione which was decomposed.

The author wishes to express her indebtedness to Dr A. J. Allen and Miss Rachel G. Franklin of this laboratory for determination of the amounts of radiation used in these experiments.

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CXCI. THE RELATION BETWEEN LIPOCHROME PIGMENTS AND VITAMIN A IN THE NUTRITION OF YOUNG FISH.

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(Received July 18th, 1933.)

INTRODUCTION.

THE large stores of vitamin A found in the liver oils of many species of fish are of peculiar interest when it is borne in mind that frequently only traces of that substance can be detected in the food on which they normally subsist.

An explanation can be found by assuming that a fish such as the cod possesses the power to synthesise the vitamin, or, on the other hand, one can believe that the reserves merely represent an accumulation; it being remembered in the first place that although very little of the vitamin may be present in the individual organisms on which they live, most fish are voracious feeders, and, secondly, that substances such as vitamin A are readily retained by the liver.

An earlier investigation in this laboratory [Coward and Drummond, 1922] indicated that the vitamin A present in the eggs of fresh-water fish (trout) disappeared during the larval period of the development of the young fish. The evidence was, however, derived from rat-feeding tests, the technique of which at that time was much less satisfactory than it is to-day. The differentiation of vitamins A and D had not been established, and it is uncertain, therefore, in the light of recent knowledge whether the conclusions reached were justified.

It must be stated that there is growing support for the view that accumulation does occur. An investigation by MacPherson [1933] of the Dominion of Newfoundland Fishery Research Station has recently indicated that a correlation can be traced between the age of fish (length) and the reserves in the liver. Similar accumulation appears to occur also in man [Wolff, 1932; Moore, 1932].

From several points of view it appeared that the changes during the early stages of the development of young fish might be studied again with advantage, primarily because it was desired to apply the modern methods of spectroscopic assay of vitamin A, but also because we had in mind the extension of this method to the lipochrome pigments which might be biologically concerned.

The relation between carotene and vitamin A in the nutrition of mammals is now generally recognised, but there are reasons for thinking that equally important relationships in which other lipochrome pigments are concerned may be exhibited by other species.

Already it is suspected that xanthophyll, which was believed to be of far less physiological importance than carotene, may have a particular function in the growth processes of the domestic fowl and possibly also of certain herbivorous species such as the guinea-pig [Rydbom, 1933].

It appeared to be necessary, therefore, to ascertain whether any of the curious and little-studied lipochromes found so widely distributed amongst aquatic species are converted into vitamin A in the tissues of fish such as the cod. A related problem, which is not only of considerable interest but also of great practical importance, is to determine whether the red lipochromes present in such organisms as *Calanus* or *Euphausia*, which incidentally are known to differ in several respects from the recognised pigments of that class, are the origin of the large amount of vitamin A found in the livers of those species of whales which feed almost exclusively on zooplankton.

EXPERIMENTAL.

A convenient material for this investigation was available in the form of the fertilised eggs of trout. Both brown trout and rainbow trout were employed. The technique of hatching the eggs and of raising and feeding the young fish was that described in the communication already referred to [Coward and Drummond, 1922]. Periodically during the larval period a number of the young fish were removed, killed and the fatty materials extracted and examined. As soon as the fry were able to take solid food they were divided into three groups and fed on the following diets:

- Group I. Pulped rat-liver.
- Group II. Finely divided fresh cod-muscle.
- Group III. Cod-muscle and fresh-water green algae.

The object of the third group was to ascertain whether the very young fish could convert the carotene or xanthophyll of the green algae into vitamin A, there being evidence that normally at this early period of their development they ingest a certain amount of "green" food. The alga employed was a motile form of *Chlamydomonas*, cultivated in tap-water¹. A culture of it was poured into the circulating water in the trout "ponds" each day at the same time as the finely divided cod-muscle was being given. Microscopic examination of fish revealed that green cells had been swallowed and digested but we had no means of judging how much was eaten. We suspect that it was quite a small amount by comparison with the other food consumed.

Again, at intervals specimens were taken for extraction and examination of the fats. In Fig. 1 there is plotted the absorption spectrum of the extract pre-

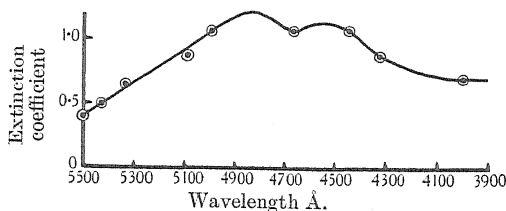


Fig. 1.

pared from the eggs of the brown trout. The absorption in the visible region does not entirely correspond with that of carotene. Saponification of the extract and application of the phase test revealed that the orange-red pigment is preferentially soluble in the light petroleum layer and, therefore, presumably it is a hydrocarbon or, at any rate, a lipochrome of low polarity.

¹ We are indebted to Prof. Salisbury for identifying the organism we employed.

A comparison of this curve with that recorded by Lovern and Morton [1931] for a pigment found in monk fish-liver oils shows a striking similarity.

The exhaustive researches of Lönnberg and Hellström [1932] on the pigments of fish suggest that this fat-soluble colouring matter may be widely distributed.

The absorption spectrum of the extract from the eggs of the rainbow trout is similar to that of the brown trout eggs, but much less pigment is present.

In Table I the absorption at 4800 Å. near the maximum of the carotenoid band and the absorption at 3280 Å. have been evaluated for the brown trout. The figures express the extinction coefficient for a concentration where 1 cc. of extract (CHCl_3) is equivalent to 1 g. of fish.

Table I.

1	2	3	4	5	6	7	8	9
Group	Days from commencement	Absorption at 4800 Å.	Absorption at 3280 Å.	Corrected value at 3280 Å.	Weight	Weight \times absorption at 4800 Å.	Weight \times corrected absorption at 3280 Å.	Column 7 + column 8
Eggs	0	2.6	2.0	0	0.083	0.215	0	0.215
Larval trout with egg sacs	6	2.2	2.08	0.39	0.091	0.200	0.0355	0.236
	15	2.2	2.10	0.41	0.096	0.211	0.0394	0.250
	23	1.8	1.80	0.41	0.108	0.204	0.0442	0.248
	30	1.1	1.4	0.55	0.136	0.150	0.0748	0.225
Feeding begun:								
Group I	37	0.98	1.96	1.21	0.126	0.124	0.1525	0.277
	51	1.12	2.08	1.22	0.104	0.117	0.1269	0.244
	62	0.70	2.10	1.57	0.140	0.098	0.2198	0.318
	76	0.325	5.20	4.95	0.165	0.054	0.8168	0.871
	92	0.82	10.70	10.07	0.162	0.133	1.6313	1.734
- A for 16 days	92	0.72	8.4	7.85	0.143	0.103	1.1221	1.225
Group II	37	0.82	1.3	0.67	0.152	0.125	0.1018	0.227
	51	1.06	1.9	1.08	0.104	0.110	0.1123	0.222
	62	0.45	1.92	1.57	0.120	0.054	0.1884	0.242
	76	0.35	1.62	1.35	0.138	0.048	0.1863	0.234
Group III	37	0.98	1.20	0.55	0.128	0.125	0.0704	0.195
	51	1.06	1.85	1.03	0.104	0.110	0.1071	0.217
	62	1.15	2.22	1.33	0.095	0.109	0.1264	0.235
	76	0.40	2.01	1.70	0.120	0.048	0.204	0.252

Mean for eggs and Groups II and III: 0.231

While the absorption at 4800 Å. is a measure of the concentration of pigment, the figure obtained at 3280 Å. must be regarded as probably compounded of three components—the absorption of any vitamin A present, the absorption due to the pigment and that due to other irrelevant chloroform-soluble materials. If the absorption of the pigment in this region is subtracted from the gross absorption, the resulting values represent changes in the content of vitamin A provided that the irrelevant substances are present in constant concentration. This latter requirement has been assumed in the results to be described, but the possibility of variations in concentration of substances other than vitamin A absorbing at 3280 Å. cannot be disregarded.

It was necessary, therefore, to allow for the absorption at 3280 Å. of the pigment, but since there was always the possibility of the presence of some vitamin A in the extracts of the eggs, an upper limit only could be set for this absorption, i.e. the extinction coefficient where the ratio of absorption at

3280 Å. to absorption at 4800 Å. is at a minimum. This condition was fulfilled in the earliest experiment on the eggs.

It will be seen (Table I) that the ratio is 0.77, so that we have assumed that at this stage the vitamin A absorption is zero, and all subsequent values at 3280 Å. have been "corrected" in a similar manner by subtracting from them 0.77 of the absorption at 4800 Å. It cannot be claimed that the "corrected" values express true absorption due to vitamin A alone, but we have eliminated by this means any variations brought about by the pigment, so that those which do occur probably arise from varying concentration of vitamin A. By multiplying this value by the mean weight of the fish, figures are obtained which represent the amounts of carotenoid (column 7) and of vitamin A (column 8) present in each fish.

In Table I it will be seen that in the young fish before feeding began the content of pigment decreased, but that this was accompanied by a corresponding rise in vitamin A content. This state of affairs continued throughout the tests on groups II and III, the two types of food showing little difference. In column 9 the sums of the values contained in columns 7 and 8 are given and these show remarkable constancy, indicating that as absorption at 3280 Å. increases, an approximately equal decrease at 4800 Å. occurs. One explanation of this would be that the pigment is being converted into vitamin A. Now $E_{1\text{ cm.}}^{1\%}$ for carotene is about 2500 at 4800 Å. [McNicholas, 1931], while for the purest vitamin A preparation $E_{1\text{ cm.}}^{1\%}$ is 1600 [Carr and Jewell, 1933]; thus we should expect for efficient conversion of carotene into vitamin A a rather greater decrease in absorption at 4800 Å. than the increase at 3280 Å. Since it is reasonable to expect that the pigment of the trout is roughly of the same absorbing power as carotene, the conversion appears to be an efficient one.

Group I fed with rat-liver showed a substantial increase both in weight and in vitamin A content as compared with the fish fed on cod-muscle. When the former were deprived of liver and were given only cod-muscle for 16 days they decreased considerably in weight and showed a reduced amount of substance absorbing at 3280 Å.

The results with the rainbow trout are expressed in the form of graphs. In Fig. 2 the superior growth on the liver diet is well marked and there is

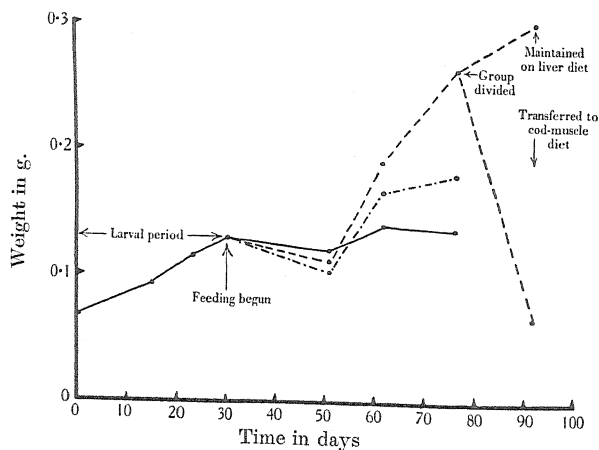


Fig. 2.

—•— Cod-muscle diet. — — Rat-liver diet. — · — Cod-muscle diet + algae.

evidence that supplementing the "white fish" diet with green algae was beneficial. From the curves in Fig. 3, there is also an indication of storage of carotene

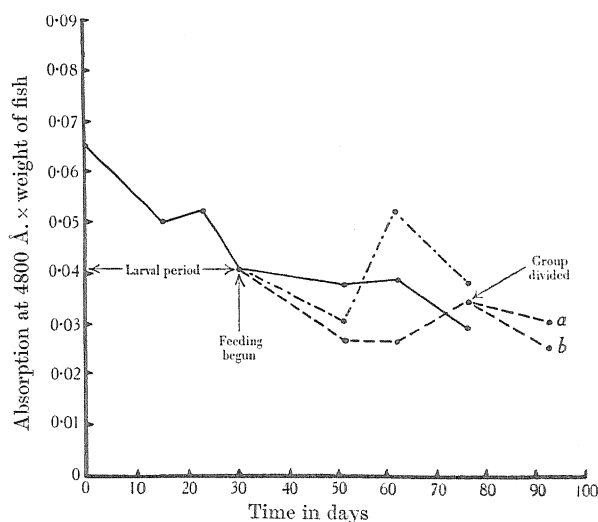


Fig. 3. (a) Maintained on liver diet, (b) transferred to cod-muscle diet.

●—● Cod-muscle diet. ●—●—● Cod-muscle diet + algae. ●—●—● Rat-liver diet.

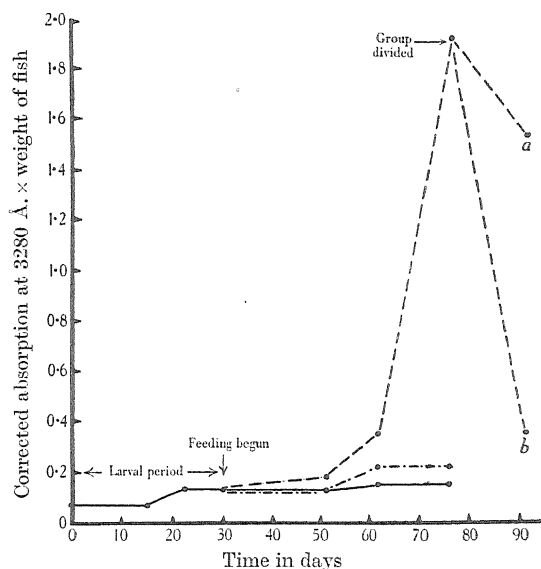


Fig. 4. (a) Maintained on liver diet, (b) transferred to cod-muscle diet.

●—● Cod-muscle diet. ●—●—● Cod-muscle diet + algae. ●—●—● Rat-liver diet.

or a similar pigment by the fish in group III. In Fig. 4 the vitamin A content in the group fed with rat-liver is shown to rise very steeply, and to fall as sharply when deprived of vitamin A for 16 days by feeding exclusively on the cod-flesh

diet. The concentration attained would appear therefore to be abnormally high. Corresponding with the rapid decline in the amount of vitamin A in the case of fish deprived of liver is a sharp decrease in weight (Fig. 2).

It will be noticed that in groups II and III the reciprocal effects of decreasing pigmentation and increasing nett absorption at 3280 Å. indicated in Table I, are also apparent (Figs. 3 and 4). It would seem, however, that the rainbow trout eggs originally possess less pigment but more vitamin A. The mean value for the constant corresponding to that in column 8 of Table I is considerably lower (0.181). For group I this figure rapidly increased until it was greater than for the brown trout. The relative vitalities of the two kinds varied in like manner. In the initial stages the rainbow trout had a high mortality but in later stages they were more hardy than the brown variety, a fact which would indicate that the "constant" referred to may be a measure of vitamin A reserves.

It is apparent that our conclusions are at variance with those derived from the previous investigation in this laboratory [Coward and Drummond, 1922] but as we have already remarked, the feeding tests which formed the basis of those experiments are open to criticism in the light of present day knowledge.

SUMMARY.

1. The ova of brown and rainbow trout contain a pigment similar to, but not identical with, carotene. It is probably a hydrocarbon¹.
2. In the development of the young fish the amount of this pigment decreases, and there is a corresponding increase of absorption at 3280 Å., suggesting a conversion into vitamin A.
3. Indications were obtained that the lipochrome content of young fish may be increased when they are fed on a diet containing a typical green alga (*Chlamydomonas*). It is uncertain whether the pigment stored is unchanged carotene or a substance showing similar absorption.

¹ [Note added in proof.] Kuhn and Lederer [*Ber. deutsch. chem. Ges.* 1933, 66, 488] have recently described a pigment "astacin" isolated from the lobster. Its formula is given as $C_{27}H_{33}O_3$ and a lactone structure is indicated by the fact that its distribution in the phase test can be reversed by changing the reaction. It is possible that this pigment is related to that we have described.

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CXCII. THE MICRO-DETERMINATION OF PENTOSE, FREE AND COMBINED. I.

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(Received July 21st, 1933.)

RECENT investigations have shown the widespread distribution of pentoses in combined form within the animal organism. In many cases the amount of material available for analysis is small and as it is frequently necessary to carry out rapidly a large number of analyses, it is advisable to have at one's disposal a simple and reliable micro-method. The majority of modern methods depend upon the conversion of pentoses into furfuraldehyde on distillation with 12-20 % hydrochloric acid [Gans *et al.*, 1888] and the determination of the amount of furfuraldehyde, either gravimetrically after condensation with phloroglucinol in acid solution or colorimetrically with aniline and acetic acid. The colorimetric method has the great advantage of detecting furfuraldehyde in extreme dilutions. Youngburg and Pucher [1924] examined carefully the conditions under which the reaction between furfuraldehyde and aniline acetate requires to be carried out in order to obtain accurate quantitative results. Pervier and Gortner [1923] had already directed attention to the advantages of steam-distillation in the removal of furfuraldehyde from the reaction mixture, and Hoffman [1927] employed this method with certain modifications, along with the colorimetric technique of Youngburg and Pucher. Steudel and Wohinz [1931] and many others have adopted, with slight modifications, the Hoffman method. Youngburg [1927] later replaced hydrochloric with phosphoric acid, removing the furfuraldehyde in the usual way by steam-distillation, and obtained the theoretical yield from *d*-xylose, 78.5 % of the theoretical from *d*-ribose, 64.9 % from *l*-arabinose and *d*-arabinose and 52.2 % from *d*-lyxose. He obtained very small yields (1-6 %) from various hexoses, disaccharides and polysaccharides, glucose furnishing 4.84 %.

The recent literature shows that even when all precautions are taken as regards removal of furfuraldehyde from the reaction mixture the yields obtained by different investigators from the same pentoses, free or combined, are far from uniform. All are agreed that the yield from the pyrimidine nucleotides is extremely small. The most recent results of furfuraldehyde yields from various purine nucleotides and nucleosides of yeast and of animal origin are those given by Steudel [1933].

The great difference in the furfuraldehyde yields between the yeast and the animal nucleotides is very striking and has naturally attracted a great deal of attention. The rise in the furfuraldehyde yield after deamination and removal of the phosphoric acid from the muscle adenylic acid has naturally been interpreted as due to a difference in the structure of the ribosephosphoric acid in the yeast and the muscle nucleotides. Embden and Schmidt [1929] were the first to show that there are striking differences between the muscle adenylic acid and the yeast

adenylic acid, among which may be mentioned solubility, optical rotation, rates of acid hydrolysis of the phosphoryl linkage, deamination (chemical and enzymic) as well as furfuraldehyde yields. Recently Klimek and Parnas [1932] have drawn attention to a difference as regards the formation of a complex copper salt with muscle adenylic acid and not with the yeast derivative.

That such differences are due in part to the position of the hydroxyl groups and the phosphoryl linkage of the pentose is extremely probable, but it is difficult to ascribe the very great disparity in the furfuraldehyde yields to such a cause.

The principle of the micro-method which we have employed during the last two years is briefly the production of furfuraldehyde from the action of acetic acid on pentoses, free and combined, in evacuated and sealed tubes kept at 170° for some hours, and the subsequent direct determination of the furfuraldehyde colorimetrically with aniline acetate. Numerous experiments were carried out in order to determine the conditions which led to maximum yields of furfuraldehyde.

(1) *Acids*. Many acids in different concentrations were tested, *e.g.* oxalic, phosphoric, acetic, acetic *plus* trichloroacetic and others. Finally it was found that acetic acid alone gave the best yields and that the most favourable concentration was approximately 50 %. This may not be the exact optimum, but such a concentration is more convenient to work with than one slightly stronger or weaker.

(2) *Temperature*. The optimum temperature was found to be about 170° in order that the maximum yield should be obtained within 2–4 hours.

(3) *Evacuation of the tubes*. Good evacuation (water- or oil-pump) is absolutely essential in order to avoid destruction of furfuraldehyde. Furfuraldehyde solutions heated under the standard conditions show no sign of any destruction, the recovery being complete.

(4) *Concentration of solutions*. Different concentrations covering a wide range were tested. Dilute solutions (1.0 cc. = 0.05 mg. furfuraldehyde) were found on the whole to be most suitable.

The technique of the method.

The aqueous solutions of the pentoses and of the purine nucleotides and their derivatives are usually made of such a concentration that 1.0 cc. of each solution should give a theoretical yield of 0.1 mg. furfuraldehyde. 50 cc. of the solution are then run into a 100 cc. flask and made up to 100 cc. with glacial acetic acid. The theoretical yield of furfuraldehyde from this 50 % acid solution is therefore 0.05 mg. per cc. Three to 4 cc. of the acid solution are then transferred with a capillary pipette into glass tubes 1 cm. in diameter and 16–18 cm. in length. The closed ends of the tubes are previously blown into small bulbs to facilitate evacuation. The tubes are then drawn out, evacuated and sealed. They are now covered with asbestos paper, placed in iron or lead tubes and heated in an electric oven at the temperatures and for the times found to be most suitable. The tubes are finally allowed to cool and opened with the usual precautions. The colorimetric determination is then made in the usual way, using as a standard the one employed by Youngburg and Pucher, the solutions for comparison being made up as follows:

I. The reaction mixture.	II. Series of fractions of standard furfuraldehyde solution (1.0 cc.—0.05 mg. furfuraldehyde).
1.00 cc. of the solution.	(1) 0.70 cc., (2) 0.80 cc., or (3) 0.90 cc. of standard furfuraldehyde.
0.5 cc. aniline.	0.5 cc. aniline.
3.5 cc. glacial acetic acid.	4.0 cc. glacial acetic acid.
Distilled water up to 10 cc.	Distilled water up to 10 cc.

After the reaction mixture and series of standards have stood for 15 minutes in the dark, they are compared, and the standard (1), (2) or (3), which most closely approximates to the colour of

the unknown is matched with it in the colorimeter. When dealing with reaction mixtures of widely varying furfuraldehyde concentrations, the final procedure of Youngburg and Pucher [1924, p. 745] was adopted in its entirety. All measurements and reaction mixtures were made with standard 1.0 cc. pipettes divided into 0.01 cc.

The stock furfuraldehyde solution is made up in water saturated with toluene so that the strength is 5.0 mg. furfuraldehyde per cc. and is stored in the ice-chest. From this the dilute standard can be prepared as required.

A few examples from a very large number are given in Table I in order to show why a concentration of 50 % acetic acid at 170° for a period of 3-4 hours has been chosen as most satisfactory.

Table I. *Yields of furfuraldehyde from pentoses, nucleotides and nucleosides under various conditions.*

Substance	Conc. of acetic acid	Temp. ° C.	% yield of furfuraldehyde after								
	%		1	1.25	1.5	1.75	2	3	4	5	6 hrs.
<i>d</i> -Xylose (<i>a</i>)*	50	170	66.5	—	—	—	84.5	86.7	92.9	93.1	80.6
" (<i>b</i>)	50	170	—	—	—	—	86.0	88.8	91.8	—	—
" (<i>b</i>)	50	180	76.4	85.1	88.9	82.2	80.5	—	—	—	—
" (<i>a</i>)	25	170	—	—	—	—	—	72.1	77.1	63.1	—
" (<i>a</i>)	75	170	50.3	—	—	—	64.5	—	—	52.7	—
<i>l</i> -Arabinose (<i>a</i>)	50	170	—	—	—	—	62.3	73.4	81.3	68.6	—
" (<i>b</i>)	50	170	—	—	—	—	69.8	70.2	70.2	—	—
" (<i>b</i>)	50	180	—	—	76.4	—	63.8	—	—	—	—
Yeast adenylic acid (<i>a</i>)	50	170	65.9	—	—	—	84.0	85.5	95.1	83.5	76.9
" (<i>b</i>)	50	170	—	—	72.6	—	81.1	82.7	81.6	71.4	—
" (<i>b</i>)	50	180	67.0	84.5	74.4	—	—	—	—	—	—
Yeast adenosine (<i>a</i>)	50	170	74.5	—	—	—	—	89.2	—	—	82.5
" (<i>b</i>)	50	170	—	—	—	—	75.0	85.6	82.1	72.6	—
" (<i>b</i>)	50	180	72.4	76.3	71.4	—	—	—	—	—	—
Yeast guanosine (<i>a</i>)	50	170	—	—	—	—	64.4	—	—	—	—
" (<i>b</i>)	50	170	—	—	—	—	70.9	75.5	—	68.2	—
" (<i>b</i>)	50	180	—	72.4	—	—	68.2	—	—	—	—
Yeast guanylic acid (<i>b</i>)	50	170	—	—	—	—	67.7	78.9	72.6	—	—
" (<i>b</i>)	50	180	72.6	72.9	71.4	—	—	—	—	—	—
Muscle adenylic acid I (<i>b</i>)	50	170	60.2	—	—	—	73.0	62.4	59.8	—	—
" II (<i>b</i>)	50	170	—	—	—	—	67.5	75.1	61.6	—	—
Ba inosinate (<i>b</i>)	50	170	—	—	—	—	76.0	—	—	—	—
Ca adenosinetriphosphate (heart muscle)	50	170	62.5	—	—	—	55.5	55.5	62.5	—	—
Ditto (rabbit's skeletal muscle)	50	170	53.8	—	—	—	53.1	49.1	—	—	—
Ditto (frog's skeletal muscle)	50	170	44.6	—	—	—	—	—	—	—	—

* (a) and (b) signify concentrations of substance yielding theoretically 0.1 and 0.05 mg. furfuraldehyde per cc. of solution.

Note. We are indebted to Dr Steudel for the adenylic acid (I), to Dr Henning for the same substance (II), to Dr Levene for the Ba inosinate and to Dr Strain for the specimen of adenosinetriphosphate from the heart muscle of the horse; to all of whom we offer our best thanks.

The furfuraldehyde yields from the muscle nucleotides are very much higher than those which have been obtained by others using the distillation method [cf. Steudel, 1933], so that differences between the nucleotides of yeast and of muscle origin are by no means so evident as one had been led to expect. Further work on this subject is being carried out.

Furfuraldehyde yields from the pyrimidine nucleotides.

A specimen of cytidylic acid supplied by the British Drug Houses Ltd., gave the following yields on heating with 50 % acetic acid at 170°.

3 hours, 27.5 %; 5 hours, 29.4 %; 7 hours, 28.2 %.

Furfuraldehyde yields from hexoses and sucrose.

In dilute solutions there was practically no furfuraldehyde production, the colour development being so slight as to make the readings impossible. In much more concentrated solutions than those used for pentoses, glucose and sucrose gave apparently small yields, 4.18 and 4.56 % respectively. It is probable however that the colour development is due to the presence of hydroxymethyl-furfuraldehyde. Slight humin formation also masks the colour change and renders colorimetric comparison unsatisfactory.

It is evident that there are variations in the maximum yields of furfuraldehyde obtained by this method, depending as always on the difficulty of obtaining maximum production of furfuraldehyde with minimum destruction, but in our opinion the same is true of all acid distillation methods. This is shown by a comparison of the yields obtained by Hoffman and Steudel respectively, using approximately the same procedure. The method here described can be readily carried out and one may easily compare and determine with accuracy the yields under exactly the same conditions from a pure pentose of known concentration and pentose in some form of combination. With the qualifications which follow from the above remarks, one may give provisionally as approximate maximum yields of furfuraldehyde from pentoses, *etc.* which have been examined the following values.

	%
<i>d</i> -Xylose	90-93
<i>l</i> -Arabinose	75-81
Yeast adenylic acid	85-95
Yeast adenosine	80-90
Yeast guanosine	70-75
Yeast guanylic acid	70-80
Muscle adenylic acid	70-75
Muscle inosinic acid	75
Muscle (skeletal) adenosinetriphosphoric acid	50-53
Muscle (cardiac) adenosinetriphosphoric acid	55-63
Cytidylic acid	27-29
Hexoses (in dilute solution)	0

SUMMARY.

A micro-method for the determination of pentoses, free and combined, is described, and the conditions under which maximum yields may be obtained are discussed.

By this method the furfuraldehyde yields from the purine nucleotides of muscle are found to be much higher than those obtained by the usual steam-distillation method from a hydrochloric acid reaction mixture.

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CXCIII. THE INFLUENCE OF COD-LIVER OIL ON THE HAEMOLYTIC COMPLEMENT OF HUMAN BEINGS.

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(Received June 19th, 1933.)

In a previous communication [Osborn, 1931], it was shown that the complement of rats fed on a diet deficient in vitamin A tended to be lower than that of control animals receiving cod-liver oil. The aim of the present work was to discover whether a dose of cod-liver oil produced a change in complement concentration in human beings. Fifty cases were experimented on, and they were compared with fifty cases receiving olive oil. A large dose of 50 cc. was given in each case in order to obtain the maximum possible effect. This was taken without difficulty by all the subjects except one. A Zulu who had a superstitious objection to fish of any sort and who detected the fishy flavour of the cod-liver oil vomited a few cc. of his dose.

In all 100 experiments were made:

- 14 on Oxford students and research workers,
- 24 on South African students,
- 42 on natives in the Johannesburg Hospital,
- 20 on natives in the City Deep Mine Hospital.

Fourteen individuals submitted to both the cod-liver oil and the olive oil, with an interval of a day or more between the experiments. In the other experiments alternate individuals were given cod-liver oil and olive oil respectively. The students were all normal healthy individuals. The hospital cases were convalescents, usually a day or two before their discharge, fractures and other simple surgical cases uncontaminated by sepsis, and others similarly free from any acute infection.

In each experiment about 0.3 cc. of blood was withdrawn from the finger tip into a small agglutination-tube between 8 and 10 a.m. The dose of oil was then given and one or more further samples of blood were taken from one to five hours after the dose.

The tubes were stoppered and allowed to stand at room temperature for 24 hours. The complement activity of the resulting serum was then estimated by measuring the time taken for complete haemolysis of a fixed volume of sensitised sheep's corpuscles by a measured volume of the serum. Two observations were made on each sample of blood. The S.A. Institute for Medical Research kindly arranged for a regular supply of the corpuscles. They were sensitised with five units of anti-sheep corpuscle serum and were suspended in normal saline to give a 3 % suspension. The details of their preparation are as follows.

2 parts of sheep's blood are drawn into 1 part of a 2 % solution of sodium citrate. The corpuscles are deposited by centrifuging and are washed three times

with 3 volumes of 0.85 % NaCl, being centrifuged for 10 minutes at 3000 r.p.m. between each washing. A volume of corpuscles, measured by means of a pipette, is then suspended in sufficient 0.85 % saline to give a 3 % suspension. To this are added 5 units of anti-sheep corpuscle serum (*i.e.* five times the minimum volume needed for complete haemolysis in the presence of complement). The mixture is then allowed to stand for at least an hour.

A capillary pipette described in the Appendix was used for the measurements. The following volumes were drawn up in the order stated with a bubble of air between each:

1.0	vol.	normal saline
1.0	„	normal saline
<i>x</i>	„	serum diluted three or four times with normal saline
1- <i>x</i>	„	normal saline
0.5	„	normal saline
0.5	„	3 % suspension sensitised sheep's corpuscles
Total		4.0 vol.

In the pipette used 1 volume was equal to about 50 mm.³ Since all the measurements were made with the same pipette, the absolute volume is of no importance. The measured volumes were expelled in the opposite order into an agglutination-tube. This procedure ensures that the serum is washed out of the pipette by two volumes of saline, and that it is separated from the sensitised corpuscles by two small volumes of saline. The solutions were mixed thoroughly, and the tube was warmed in a glass-sided water-bath at 37°. A white glazed tile, ruled with black lines, was placed behind the tubes in the bath, and they were illuminated with a strong beam of light. In this way it was possible to estimate, to within a few seconds, the point of complete haemolysis. Since a reaction taking longer than about 4 minutes has a less definite end-point, the dilution of the serum and the volume of the dilute serum used (*x* in the list of reagents), were adjusted to give a haemolysis time of 2-3 minutes.

The activity of the complement after the dose was expressed as a percentage of the strength before the dose.

$$\begin{aligned}
 & \frac{\text{Activity of complement after dose}}{\text{Activity of complement before dose}} \times \frac{100}{1} \\
 &= \frac{\text{Speed of haemolysis after dose}}{\text{Speed of haemolysis before dose}} \times \frac{100}{1} \\
 &= \frac{\text{Time for complete haemolysis before dose}}{\text{Time for complete haemolysis after dose}} \times \frac{100}{1}.
 \end{aligned}$$

For these times to be comparable it was obviously necessary for the same volume of serum to be used in the estimation of the activity before and after the dose, *i.e.* in any one comparison the dilution of the serum and the volume of the diluted serum (*x*) were the same.

In a few of the cases samples of blood were taken hourly from 1 to 5 hours after the dose, in others a single sample was taken at from 2½ to 4 hours. In all 174 estimations were made following cod-liver oil and 170 following olive oil. The results are expressed in the form of a histogram in the accompanying diagram. 100 %, marked by the heavy black line, is the strength of the complement before the dose.

Protocol of one pair of observations:

Subject: J. H. G.

Date: 18th Jan. 1933.

Blood sample taken at 9.30 a.m., dose of 50 cc. of cod-liver oil given immediately after, second sample taken at 1 p.m., food and violent exercise being avoided in the interval. Blood stood at room temperature overnight to allow separation of the serum. Serum diluted with 3 volumes of 0.9 % saline.

	9.30 a.m. blood	1 p.m. blood
Volume diluted serum	0.6	0.6
Volume sensitised corps.	0.5	0.5
Volume 0.9 % saline	2.9	2.9
Haemolysis time	168 secs.	145 secs.
Percentage of original speed	—	$\frac{168 \times 100}{145} = 116 \%$

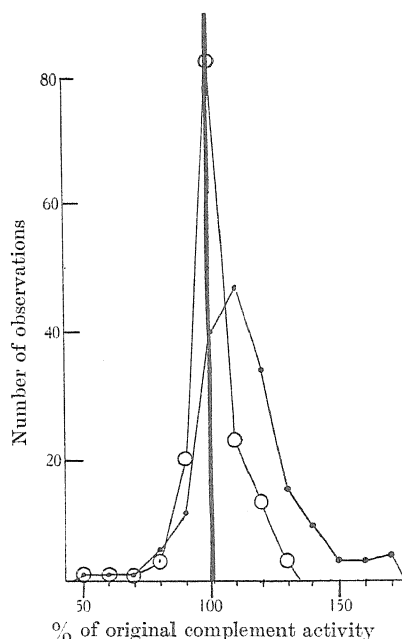


Fig. 1. Histogram showing complement activity of serum of individuals after taking cod-liver oil and olive oil. Expressed as percentage of activity of serum taken before dose.

The statistical analysis of the figures gives the following results:

Mean of cod-liver oil	= 113.4
Mean of olive oil	= 102.7
Difference	= 10.7

Standard deviation of cod-liver oil ($\sigma_{c.l.o.}$) = 19.0

Standard deviation of olive oil ($\sigma_{o.o.}$) = 10.8

Standard error of difference ϵ_{12}

$$= \sqrt{\frac{\sigma_{c.l.o.}^2}{N_{c.l.o.}} + \frac{\sigma_{o.o.}^2}{N_{o.o.}}} = 1.66.$$

Since the deviation of the means is approximately 6.5 times the standard error of the difference, it seems to be significant.

DISCUSSION.

It is an accepted fact that haemolytic complement is not a single substance. The work of Buchner [1893], Ferrata [1907], Sachs and Teruuchi [1907], Brand [1907], and others showed that it contained two thermolabile parts, the "Midstuck" and "Endstuck." The work of v. Dungern [1900], Ehrlich and Sachs [1902], Coca [1914], Hyde [1925] and others led to the recognition of a third part, while Gordon, Whitehead and Wormall [1926] produced good evidence of a fourth part. In these circumstances it would not be expected that an increase in the concentration of one of the parts would invariably lead to an increase in the total complement activity. This would only occur if the part increased were the limiting factor in the blood in question. This supposition may explain the unevenness of the results obtained. While the administration of cod-liver oil caused an increase as compared with the olive oil when measured by the average effect, and while this increase was well marked in a percentage of cases, it did not occur invariably. This is what might be expected by our previous reasoning. If some factor, present in cod-liver oil and absent from olive oil, caused an increase in one of the parts of the complement complex, an ingestion of complement would cause an increase in the total complement activity in some cases but not in others.

These results are interesting when taken in conjunction with the previous results [Osborn, 1931], which showed that rats fed on a diet deficient in vitamin A had, on the average, a lower complement than control rats receiving cod-liver oil.

SUMMARY.

The ingestion of a dose of cod-liver oil by 50 experimental subjects produced, on the average, a rise in haemolytic complement which was greater than that produced by olive oil. The significance of the fact that this rise took place in some cases but not in others is discussed.

The author's thanks are due to Prof. J. P. Dalton, who kindly undertook the statistical analysis of the results, to Dr G. Buchanan of the S.A. Institute for Medical Research for the supply of blood corpuscles, and to the subjects who allowed a rather unpleasant experiment to be performed on them.

APPENDIX.

The instrument described below has been found to be suitable for the repeated measurement of volumes of liquid of the order of 5 mm.³

A piece of tubing is drawn out into the shape shown in Fig. 2. A small drop of mercury, equal in volume to the smallest graduation required, is introduced at the broad end *D* and allowed to run round into the capillary *A*. A rubber teat is filled with mercury and pushed over the broad end *D*. Pressure on the teat is regulated by means of a screw-clip and in this way the column of mercury in the capillary *A* may be moved down the tube and the required number of graduations marked on the tube in Indian ink or glass paint. The instrument is then clamped in a vertical position. A second clamp holds the rubber teat. Liquids are drawn into the pipette by relaxing the pressure on the teat and expelled from it by increasing the pressure.

For accurate measurement the elasticity of the air within the instrument is a disadvantage and therefore the volume of this air is reduced as much as possible. It is for this reason that *C* is drawn out into a capillary and that the teat is filled with mercury.

In making mixtures of different volumes a method is used which is described by Wright. The measured volumes are separated by columns of air in the capillary. If, for instance, it is required

to mix 1.7 volumes of *A* with 0.6 volumes of *B* by means of a pipette holding 1 volume and graduated into 10 divisions, the following are drawn up into the pipette:

1.0 volumes *A*,
a small column of air,
0.7 volumes *A*,
a small column of air,
0.6 volumes of *B*.

These are then all expelled together. They may be mixed by being repeatedly drawn in and out of the pipette. Another convenient method is to blow a small bubble of air into the mixture and rotate it about.

The small bulb *B* prevents liquids being drawn over into the mercury in *D*.

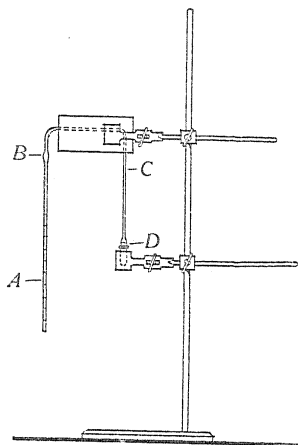


Fig. 2. Diagram of pipette.

Wright [1921] advocates the coating of the inside of small pipettes with wax to obviate the error produced by wetting the glass. The author has not, up to the present, found a wax which will hold to the walls for any length of time. If such a substance could be found, it would be a great advantage in all volumetric micro-chemical work. An alternative suggestion of Wright's has, however, been found to be helpful. If the external surface of the tip of the pipette has a coating of wax the removal of the last drop of liquid is facilitated.

In most cases one pipette would be used for all measurements, therefore the absolute volume of the graduated part of the pipette need seldom have any definite value. Provided it is divided into a number of equal parts, relative volumes can be measured accurately.

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CXCIV. HIGH PROTEIN DIETS AND ACID-BASE MECHANISM.

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(Received July 22nd, 1933.)

ALTHOUGH it has been widely held, particularly since the experiments of Chittenden and of Chalmers Watson, that the presence in the food of high proportions of protein is harmful, there is still much conflict of evidence as to whether this is really so in the case of omnivorous species. Many investigators have attempted to analyse the underlying effects of increased protein rations, particularly with regard to the effect of protein in producing kidney disorders. The primary reason for undertaking the following experimental work was to determine whether the adverse effect which has been reported by some investigators was due to the acidogenic properties possessed by protein in virtue of its content of sulphur and phosphorus². This acidogenic effect has been demonstrated in man by Farquharson *et al.* [1931] who found increased excretion of ammonia and loss of calcium in response to a high protein diet. As to other effects of high protein diets in man, though such diets tend to alter the proportion of putrefactive organisms in the intestine [Sanborn, 1931; Torrer and Montu, 1931], the work of Krogh and Krogh [1913] and Thomas [1927] on eskimos, and that of McClellan and DuBois [1930], McClellan *et al.* [1930], and Tolstoi [1929] on trained subjects, all fail to indicate serious harmful effects from excessive dietary protein, whilst physical inferiority results in the case of African and of Bengali tribes if the protein is low [Orr and Gilks, 1931; McCay, 1912].

In animals the presence of excess of protein in the food has been found to cause renal hypertrophy by many workers [Osborne *et al.*, 1926-27; Hassan and Drummond, 1927; Reader and Drummond, 1925; 1926; Drummond *et al.*, 1922; Jackson, 1924-25; Jackson and Riggs, 1926; Francis *et al.*, 1931; Stewart, 1929; Longwell *et al.*, 1932; MacKay *et al.*, 1928]. Actual renal damage has been observed by Newburgh *et al.* [1919; 1923; 1928; 1931], Polvogt *et al.* [1923], Blatherwick *et al.* [1931], Maclean *et al.* [1926], Evans and Risley [1925], Smith *et al.* [1927], Jackson and Moore [1928] and Parsons [1931]. Newburgh and Curtis [1928] found that protein in the form of dried liver was particularly harmful; on the other hand Newburgh and Marsh [1925], who observed renal lesions after intravenous injection of certain amino-acids, were unable to ascribe the toxic effect to any particular compound.

According to Drummond and his colleagues and Hartwell [1928] high protein diets decrease the growth-rate of rats, whilst the reverse was claimed by Osborne and Mendel [1924]; Slonaker [1931] differentiated between the unfavourable effect of high protein intake on activity, reproduction and life-span

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² In the complete oxidation of 100 g. of average protein with N : P ratio 17 and N : S ratio 14.5, phosphoric acid is produced equivalent to 30 cc. N alkali and sulphuric acid equivalent to 50 cc. N alkali.

and its favourable effect on lactation, growth of the young and freedom from tumours.

The question arises whether the discrepancies among these findings can be explained, in part at least, by the differences in the acid-base balance of the diets used by the various workers. Thus, Osborne *et al.* [1926-27] supplied vitamin B partly in the form of alfalfa which would raise the allowance of fixed base. The evil effect of dried liver might be explained by its potential acidity, whereas the protective action of unheated liver could possibly be ascribed to one of the constituents of the vitamin B complex. It was considerations such as these which led to a re-investigation of the relationship of protein, acid-base balance and vitamin B with regard to impaired growth-rate and albuminuria. It is interesting to observe that Watson [1910] found softening of the bones of his experimental rats when he fed them on meat diets without any additional alkali. These changes suggest that, in the absence of basic substances from the diet, the calcium of the bones was mobilised for purposes of neutralisation. Presumably, carnivorous animals under natural conditions cover the acid-producing elements of their meat diets by eating bones.

The rôle of common dietary salt mixtures in relation to the acid-base mechanism had been touched upon by Hassan and Drummond [1927], who found that when the growth-rate of rats was retarded by diets containing a large proportion of protein, autoclaved marmite was protective, while the ash of marmite offered no protection against the inhibitory effect on growth. A special nephrotoxic action of cystine has been assumed by Cox *et al.* [1929] and others who have observed an acute toxic nephrosis to occur when cystine is added to the food of young rats. The addition of 0.1 g. cystine means a virtual increase of 1.6 cc. of *N* acid. The question of cystine nephrosis has formed the subject of another paper [Bell, 1933, 1].

We have been in the habit of thinking that the acid-base mechanism of most animals (the rabbit, among laboratory animals, being an exception) is extraordinarily efficient, and that ammonia can readily be utilised when fixed base is deficient. However, Farquharson *et al.* [1931] report that, whenever there is a rise in the ammonia excretion there is simultaneously an increased loss of calcium.

EXPERIMENTAL.

Experiments were planned to determine whether the following diets could be improved by the addition of a mixture of sodium citrate and potassium carbonate:

1. Diets containing 70 % caseinogen.
2. Egg-white diets: (a) containing 20 % egg-white, (b) containing 66 % egg-white.

1. Diets containing 70 % caseinogen.

A. Three dozen rats were used in the first series to ascertain whether the previous experiences of this laboratory could be confirmed, *i.e.* that rats ingesting large amounts of caseinogen exhibit subnormal growth, but that the inclusion of an additional ration of yeast overcomes this retardation of growth. Three groups of rats received the following diets respectively:

	X	Y	Z
"Light white casein"	20	70	70
Dried brewer's yeast	5	5	15
Rice starch	68	28	28
Cod-liver oil	2	2	2
Salt mixture (McCollum's)	5	5	5

The average growth curves (Fig. 1) demonstrated that the diet containing 70 % caseinogen with 5 % yeast had an adverse effect on growth, but that a decided improvement occurred with 15 % yeast.

B. Several groups of rats, carefully chosen litter-mates, received diets Y and Z as above, but were further subdivided so that half of them were given enough of a potentially alkaline mixture to reduce the acidity of their urine almost to zero. The mixture of salts contained sodium citrate (10 parts) and potassium carbonate (7 parts); excessive alkalinity was avoided for fear of damaging the kidneys thereby; the urine, therefore, was kept just acid. The graphs in Fig. 2 *a* show that no improvement occurred when the alkali was added; growth of rats ingesting diet Y was still subnormal, though, probably through some seasonal variation, this set of rats grew rather more vigorously than those of Fig. 1. It appears, therefore, that 5 % McCollum's salt mixture in the diet is adequate to meet the need for fixed base when the proportion of caseinogen in the food is 70 %.

Although the majority of the animals studied in this series gave us no reason to believe that the subnormal rate of growth encountered when the diet contained 70 % caseinogen could be attributed to the acidity, one anomalous result must be mentioned in which a number of rats, obtained from a dealer, behaved rather differently. Those being fed on diet Y without alkali grew more slowly than those consuming the extra mixture of potentially alkaline salts. But as this result could not be repeated, we were compelled to regard it as purely fortuitous.

Incidentally, the graphs in Fig. 2 *b* show that the addition of alkali to a 70 % caseinogen diet containing 15 % yeast does not enhance the growth still further; nor does it produce supernormal growth when compared with the rate of growth when caseinogen constitutes 20 % of the diet.

2. Egg-white diets.

Confirmation has been obtained of the observations of Bateman [1916], Boas [1924; 1927], and Parsons [1931] that uncoagulated egg-white in the food of rats rapidly causes diarrhoea which is fatal if the diet is not amended.

In the experience of Boas with 20 % egg-white, coagulated and dried in an oven, then added to the food, rats grew normally at first, then declined and died; against this adverse effect, certain varieties of starch were protective. In the following experiments, carbohydrate was therefore supplied in the form of cane sugar, as had been done by Parsons. Chinese egg-white was prepared in one of two ways—it was coagulated and finely minced; or coagulated, dried at 80° and finely ground. Using the egg-white prepared as above, diets were compounded of:

- 20 % egg-white (dry basis),
- 68 % cane sugar,
- 5 % dried brewer's yeast,
- 5 % McCollum's salt mixture,
- 2 % cod-liver oil.

The severe effect reported by Boas failed to occur. There was, however, diminished growth-rate compared with that of animals ingesting food containing 20 % caseinogen (Fig. 3). No improvement was induced by adding alkali to the food. Hence, the depressed growth rate resulting from diets containing 20 % egg-white cannot be attributed to the acidogenic properties of the cystine in which it is rich.

We also repeated the experiments of Parsons, using 66 % egg-white (dry basis), with and without potential alkali, and found that no amelioration took

place when the diet included the extra base (Fig. 4). The finding that 5 % McCollum's salt mixture was adequate to cover the acid produced by the meta-

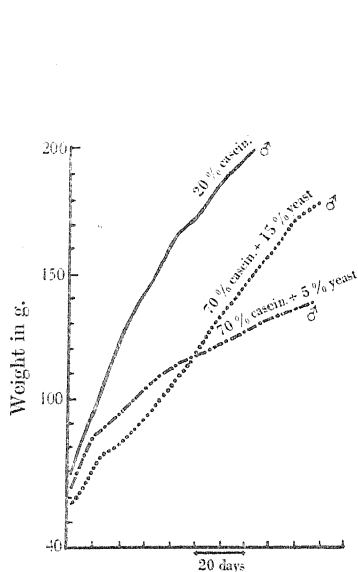


Fig. 1.

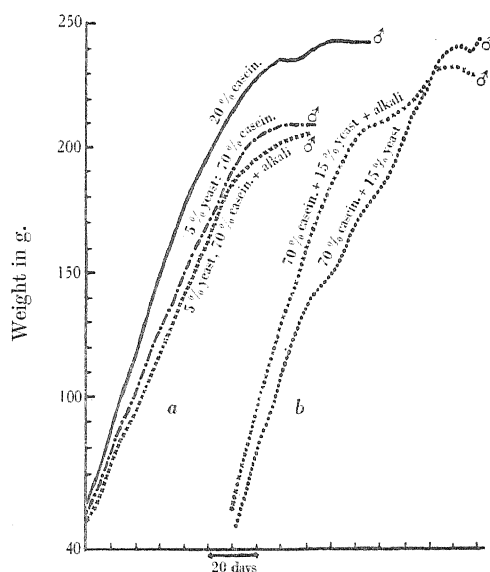


Fig. 2.

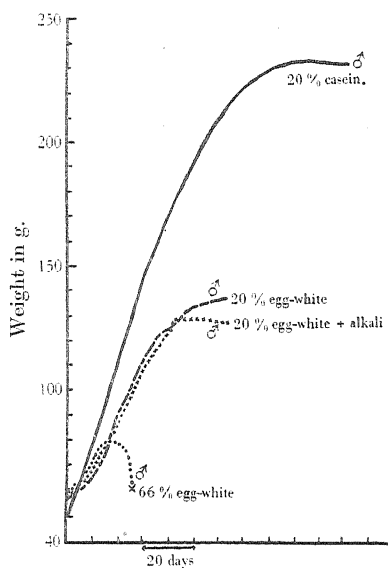


Fig. 3.

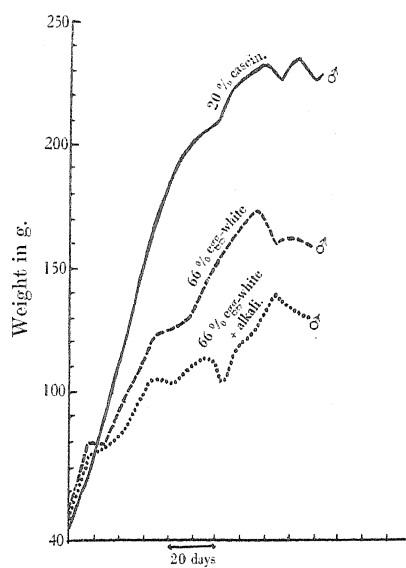


Fig. 4.

bolism of 66 % egg-white in the food thus dismissed the original idea of the research. What follows, therefore, is a digression in which egg-white diets are discussed.

Not only did we use egg-white, coagulated and dried at 80°, according to Parsons' method of preparation, but also egg-white coagulated and minced. Both Chinese and fresh egg-albumin gave, as Parsons found, comparable results. Care was taken not to overheat the egg-white. The food consisted of:

66 % egg-albumin (dry basis),
22 % cane sugar,
5 % salt mixture (McCollum's),
5 % dried brewer's yeast,
2 % cod-liver oil.

With our experiments, one group of rats exhibited considerable resistance to the diet; this group, charted in Fig. 4, failed to develop the acute nephritis which Parsons had observed; they grew comparatively well, and most of this group did not get the pellagra-like condition which she described. Two individual rats, however, which merely maintained their weight at about 60–80 g. for twelve weeks, were affected with the scaly and gangrenous condition of the tail, which has now been observed on a variety of diets, and which, in this laboratory, we are inclined to attribute to chronic malnutrition arising from the absence of one of the vitamin B factors.

Another set of animals, however, also receiving the 66 % egg-white diet, manifested at an early stage the symptoms of acute nephritis as described by Parsons, and most of them died (Fig. 3). The urinary findings included albuminuria, renal cells, epithelial casts, red blood cells; and in addition determinations of the blood-urea gave the high average figure of 131 mg. per 100 cc. blood. Consistently with this, the "clinical" symptoms were drowsiness and anuria, while the kidney sections showed acute nephritis. But the feature which we thought significant about the results was that one or two animals recovered without any alteration in the diet—a fact already observed by Parsons in the case of egg-white diets; similar recovery has also been reported by Cox, Smythe and Fishback in the case of cystine nephrosis. The kidney symptoms of such rats as did not die cleared up, and the animals continued to maintain their weight. The idea became insistent that the probable explanation of the train of events was that a septicaemia had occurred; that the kidneys had been incidentally affected; and that when an immunity had been established, recovery took place. An attempt was made to determine whether there was a blood infection; seven of the affected animals were killed with chloroform, and blood cultures were made, five of which were sterile, one grew a streptococcus, another grew a coliform bacillus. The results were therefore inconclusive. A possible explanation of this hypothetical septicaemia was sought in the altered conditions in the intestine, as a breeding ground for bacteria. It will be seen that the animals which were doing comparatively well on the 66 % egg-white diets exhibited irregularities in their weight curves (Fig. 4); and moreover we observed that they were subject to slight, intermittent diarrhoea. The possibility suggested itself that, for some unexplained reason, egg-white was perhaps less readily digested than other proteins, and that this might make for a greater percentage of protein degradation products in the intestine. It became interesting, therefore, to determine the percentage of nitrogen in the faeces, and it was found that excreta collected just before the rats developed nephritis contained approximately 0.06 g. N per g., whereas animals eating the stock diet were excreting 0.02 g. N per g. faeces. Later, in animals which had recovered, the quantity of nitrogen being excreted from the intestine became less—*viz.* 0.03 g., presumably as the

intestine responded by increased activity. Similar estimations of nitrogen on the excreta of rats ingesting 70 % caseinogen gave the average figure of 0.03 g.

With the presence of excessive nitrogenous material in the gut, there was the likelihood of increased numbers of putrefactive organisms in the intestine. The urine was found to give a highly positive test for indican as compared with the urine of rats being fed on the 70 % caseinogen diet. Even the rats which were doing comparatively well on the egg-white mixture always gave a strongly positive test for indican. Possibly, this gives a clue to the severity of the results of an egg-white diet as compared with other high-protein diets. The underlying effect is perhaps the presence of substances like cystine which alter the oxidation-reduction potential of the gut, and so favour the growth of specific types of organisms. Work on *in vitro* cultivation of anaerobes by Lepper and Martin [1929] has demonstrated that, for example, the addition of cooked muscle to culture medium favours the growth of anaerobes. It seemed reasonable to infer that the severe diarrhoea attendant on the administration of uncoagulated egg-albumin was only an exaggeration of this state of affairs; that since raw egg-white is not readily acted on by pepsin-HCl, as Bateman observed, there is an increased chance for bacterial multiplication in the intestine. Feldman [1920] quotes Dementjeff as stating that giving the raw whites of two eggs to a baby resulted in the appearance of indican in the urine. Recently, Nedzel and Arnold [1930-31] have shown that in the presence of uncoagulated egg-white, the permeability of the intestine to *B. prodigiosus* is increased.

Urinary examinations.

During the course of these feeding experiments, the rats were put into metabolism cages for the purpose of testing their urines for "total acidity" and for albumin. It was observed from estimations of acidity and ammonia that the rat is well able to utilise ammonia as a base. It was also obvious that considerable amounts of calcium could be absorbed into the blood stream and excreted by the kidneys; this was true whether the calcium was given as lactate or carbonate. Thus, when some stock rats were being compared with animals on the experimental diets, it was found that calcium carbonate became deposited on the funnel and urine trap, and the urine, even in the presence of toluene, became alkaline on standing. Presumably, this calcium was excreted as the bicarbonate. That rabbits can absorb calcium given as carbonate has been demonstrated by McGowan *et al.* [1931], who incorporated it in the diet and found that the rabbit's urine after standing contained a deposit of calcium carbonate. This ability of the rat and of the rabbit to absorb calcium given as carbonate from the alimentary tract seems worth mentioning in view of the belief commonly held that human beings have difficulty in absorbing calcium.

Tests for albumin in the urine revealed the fact that mature male rats normally excrete about 0.5-0.8 g. albumin per litre of urine, but that the urine of females is generally free from albumin. The results of a study on this point have been published in a separate note [Bell, 1933, 2]. Females ingesting the diets which contained a large proportion of caseinogen excreted no albumin; and no increase occurred under these conditions in the amount excreted by the males. From urinary examinations no evidence of kidney derangement was obtained.

Estimation of blood-urea.

After the animals had been fed for six months on the diet containing 70 % caseinogen, their fasting blood-urea was determined; they were fasting for six hours before being killed with chloroform. The average blood-urea for the

group receiving additional alkali was 39.9 mg. per 100 cc.; for those without alkali, the average was 41 mg.; for a control group of whose food the protein constituted 20 %, the average was 42 mg. Blood analyses by Jackson [1924-25], and by Osborne *et al.* [1926-27] showed that rats receiving high protein diets had a higher level of non-protein-nitrogen and of urea than rats ingesting normal amounts of protein; their animals were presumably not fasting. Addis, MacKay and MacKay [1926-27] have already demonstrated that the non-protein-nitrogen level quickly falls to normal when food is withheld; this extra nitrogen appears to be due, therefore, to the presence of large amounts following absorption rather than to the inability of the kidney to excrete it.

Histology.

Examination of the kidneys of rats which had received food containing 70 % caseinogen for six months failed to reveal any pathological changes. According to Newburgh, it requires a longer period than six months' feeding with excessive caseinogen to produce degenerative changes.

SUMMARY.

1. The experience of those workers is confirmed who find that rats exhibit a subnormal rate of growth when fed on diets consisting largely of caseinogen and containing the usual proportion of yeast serving as the source of the vitamin B complex, and who also find that the inclusion of further proportions of yeast supplement renders the high-caseinogen diet adequate for growth.
2. The addition of potential alkali to diets containing a large percentage of caseinogen or coagulated egg-white does not improve the growth-rate. The presence of 5 % McCollum's salt mixture in the diet is therefore sufficient to meet the need for fixed base when high protein diets are given.
3. No evidence of kidney damage has been obtained in rats which have been fed for six months on diets containing 70 % caseinogen.
4. The presence of uncoagulated egg-white in the diet of rats is rapidly fatal, as has been found by Bateman and others.
5. Subnormal growth occurred as a result of feeding rats with diets containing 20 % coagulated egg-white, but the decline in weight reported by Boas failed to occur.
6. Subnormal growth also resulted when the food included 66 % egg-white; some of the animals developed acute nephritis and died. The findings of Parsons are therefore confirmed.
7. Examination of the faeces for nitrogen and of the urine for indican in the case of animals receiving egg-white diets led to the belief that excessive putrefaction in the intestine occurs when these diets are given.

The author wishes to express her grateful appreciation to the Royal Society of Medicine for the scholarship which has made these studies possible; to the Medical Research Council for a grant to cover expenses of materials; and to Prof. J. C. Drummond for helpful advice and criticism.

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CXCV. CONDITIONS OF HYPERTROPHY OF THE SEMINAL VESICLES IN RATS.

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(Received August 27th, 1933.)

PART I.

VOSS AND LOEWE [1931] and Moore and Gallagher [1930] have described certain restoration phenomena in secondary sex organs of castrated mice and rats respectively, by treatment with male hormone, the former authors being the first to use changes in the epithelium of the seminal vesicles as a rapid cytological test for the action of the hormone. Complete restoration was claimed by Moore and Gallagher [1930] to result from administration of 5 capon units (as defined by Gallagher and Koch [1930]) daily for 21 days, this daily dose being equivalent to 7.5 capon units as defined by Freud *et al.* [1932].

As will appear from the work to be described below, the male hormone is only one of the hormones which act on the seminal vesicles, of which it causes the epithelium to hypertrophy; the complete restoration claimed by the above mentioned authors is impossible with male hormone alone and was probably obtained by the use of preparations contaminated with oestrin or its homologues, these substances alone being stimulants of smooth muscular hypertrophy.

In this laboratory particular attention has been paid to parallel experiments on capons and on rats. The reaction of capons to preparations of differing purity and obtained from various sources proved to be reliable in our hands, contrary to statements of Korenchevsky *et al.* [1933], who reject the capon method as unreliable, and advocate testing on rats. To their statement the same criticism applies as in the case of Moore and Gallagher [1932].

In capons general conditions such as sunlight, health of the animals and, according to de Fremery [1931], thyroid function during various seasons as well as the male hormone influence comb growth. It must be especially pointed out in this connection that ketohydroxyoestrin (menformon) in crystalline form up to 1000 M.U. a day has no influence whatsoever upon comb growth. The seminal vesicles on the other hand are distinctly influenced by menformon or its homologues and as will be shown in Part II, these substances may be important in facilitating the action of male hormone at comparatively low doses of the latter.

On account of the irregular response of the seminal vesicles to various preparations, it was impossible to establish a constant ratio of minimum active quantities of male hormone between rats and capons. Continued fractionation of extracts led to their gradual loss of potency with reference to seminal vesicles without corresponding loss of potency in capons. The preliminary assumption of a separate comb growth and seminal vesicle hormone was soon replaced by another conception, which will be presented below.

Choice of material.

Seminal vesicles are easy to prepare and they respond rapidly to castration or treatment. They may serve as indicators of the hormonal state of the genital system, although there are other organs, such as the preputial glands of rats, the reaction of which is not conditioned by the presence of some other active substance though it is less specific than that of seminal vesicles. With these organs we propose to deal later.

The weight of the seminal vesicles of adult rats ranges between 120 and 800 mg. varying with (1) secretory state, (2) size of testicle, (3) structure of testicle, (4) size of hypophysis, thyroid and adrenals, (5) general condition of the animal. After castration of adult rats the weight of the seminal vesicles varies from 90 to 350 mg. The atrophy does not reach a definite basic point even after months. There is a marked difference between rats castrated during infancy or later; in the former the seminal vesicles cease to grow at once after removal of the testicles. Whilst a complete restitution of the comb and other male characters was obtained in capons by continued treatment with male hormone [Freud *et al.*, 1932] it is difficult for male hormone to act on completely atrophic seminal vesicles, just as progesterin [Corner and Allen, 1929] is potent only after preparation of the muscular tissue of the uterus of infantile rabbits by oestrin. The seminal vesicles of rats castrated during infancy may be taken therefore as indicators, not of male hormone only, but of complete testicular activity. This idea is supported by experiments on senile and on non-castrated infantile male rats with gonadotropic substances, which, stimulating the whole of the testicle, lead to an almost complete (precocious) development of the seminal vesicles [de Jongh, 1930].

On these grounds we decided to use infantile rats as test material and simplified the method by beginning their treatment almost immediately after castration, instead of awaiting maturity. In these animals we aimed at forcing a precocious development of secondary sex characters. Under these circumstances the weight of the seminal vesicles is the first indicator of the effect of a certain treatment, the histological picture being used for the analysis of their growth.

Technique.

25–45 g. (3–5 weeks old) rats are castrated by the transscrotal route. There is no bleeding; one stitch closes the wound. This operation has the object of preventing spontaneous genital development during the experiment. Three days later a course of 8 injections is started, litter-mates as far as possible being equally distributed between experimental and control groups. Twice a day 0.1 cc. liquid is administered subcutaneously. On the day following the last injection the animals are killed with chloroform. Both seminal vesicles are cut out in one semilunar piece and weighed on a torsion balance immediately after removal. A narrow bridge of prostate tissue connects the two lobes. Formaldehyde is used for fixing and haematoxylin-eosin for staining.

Histology of seminal vesicles.

Seminal vesicles have a peritoneal covering, connective tissue stroma with smooth muscle tissue imbedded therein, a small zone of subepithelial stroma and a single layer of cylindrical epithelial cells lining a sinuous cavity. In moderate hypertrophy the sinuosities are macroscopically visible.

Hypertrophy. Muscle cells large, with bright nuclei. Frequent mitoses. Epithelium high cylindrical, with basal nuclei reaching to 1/3 or 2/3 of the total height of the cells and leaving

a protoplasmic margin free towards the cavity. Mitotic cell divisions characterise the beginning of hypertrophy, they diminish later on. The cavity contains basophilic, homogeneous secretion.

Atrophy. Small muscle cells with dark nuclei, low epithelium of cubical cell form, without protoplasmic margin, the nuclei filling the cell bodies almost completely. No mitoses, only sporadic pycnosis of nuclei, imitating cell division.

Muscular hypertrophy does occur without epithelial development, while epithelial hypertrophy without a certain muscular development was never observed.

Apparent paradoxical effect of menformon.

In a previous paper [Freud *et al.*, 1933] muscular hypertrophy of seminal vesicles under the influence of menformon (ketohydroxyoestrin) was described under this heading. Male and female hormones are produced in both sexes in approximately equal quantities, as judged by their excretion in the urine of males and non-pregnant females. The two substances are distinctly different pharmacologically, they act on different tissues. The effect of menformon in males is only apparently paradoxical.

Weight of seminal vesicles.

In Table I 32 untreated castrated male animals are classified according to body weight and weight of the seminal vesicles.

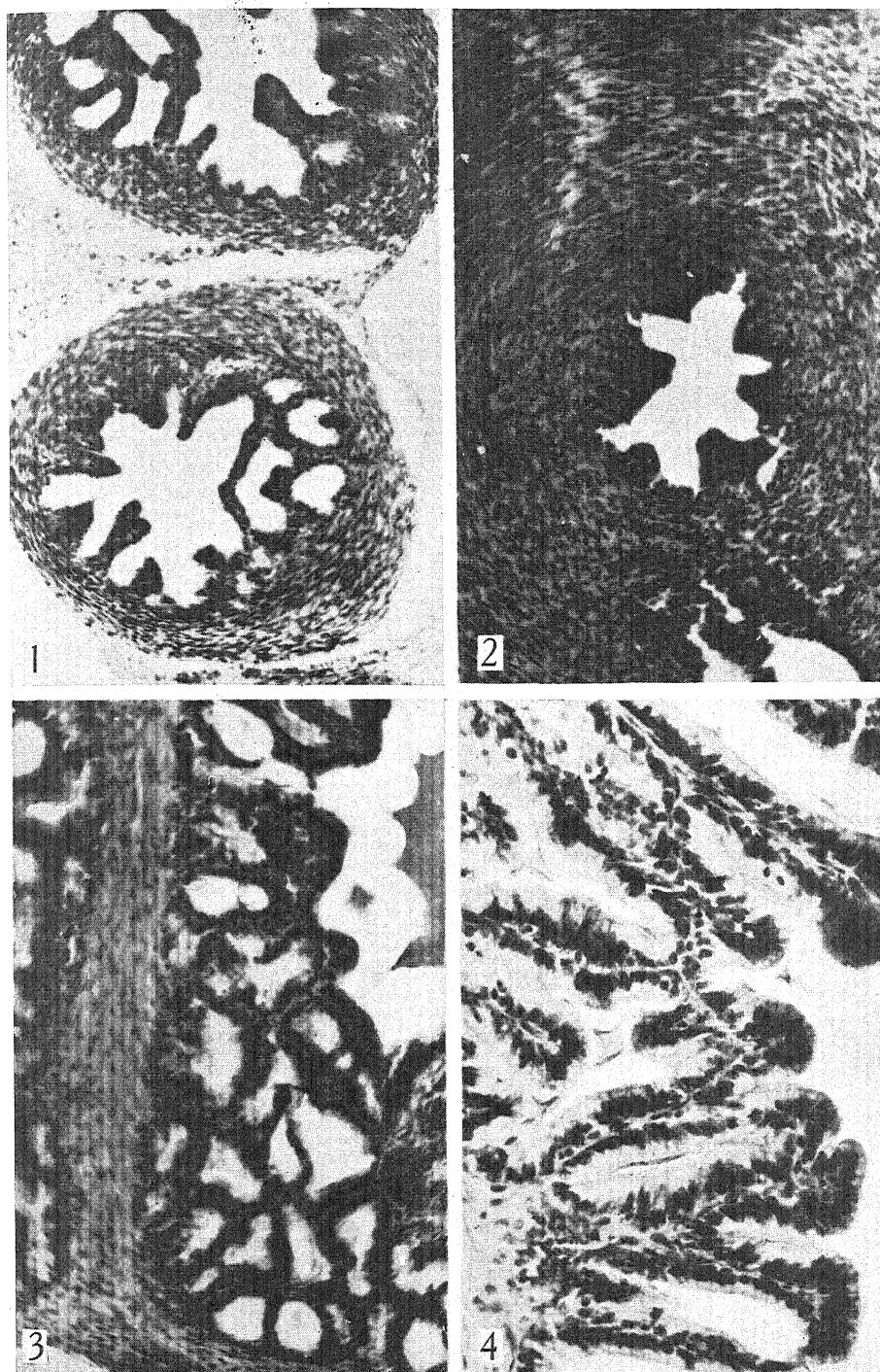
Table I.

Body weight g.	Weight of seminal vesicles (mg.)						Total
	3-4	4-5	5-6	6-7	7-8	8-9	
21-25	—	2 (Feb.)	1 (Feb.)	—	—	—	3
26-30	1 (May)	1 (July)	—	—	—	1 (July)	3
31-35	—	—	—	—	—	—	—
36-40	1 (July)	1 (May)	1 (Jan.)	1 (July)	2 (July)	—	3
41-45	—	—	1 (May)	1 (Mar.)	2 (Jan.)	—	—
46-50	—	—	—	2 (May)	2 (Feb.)	1 (May)	1 (Feb.)
51-55	—	—	—	1 (Feb.)	1 (Feb.)	—	—
51-55	—	1 (Feb.)	—	1 (July)	1 (May)	1 (Mar.)	—
51-55	—	—	—	—	1 (Feb.)	1 (July)	—
Total	2	5	3	6	10	4	2
							32

From this table we may conclude that seminal vesicles weighing more than 10 mg. scarcely ever occur in animals of less than 55 g. Wallen-Lawrence and van Dyke [1931] state that infantile non-castrated rats below 40 g. body weight have seminal vesicles of an average weight of 9.24 mg. Our material with reference to non-castrated rats confirms this statement. Geographical and racial differences do not seem to have any noticeable influence. The seminal vesicles of castrated and of intact infantile males between 25 and 45 g. body weight practically do not differ. Histologically no large difference exists either, with the possible exception that nuclear pycnosis occurs in castrates only.

How does male hormone act?

Assuming that the histological state of the epithelium of the seminal vesicles decides whether a male hormone preparation is potent or not, the question of the relation between weight and histology arises. The degree of epithelial response may be classified according to two criteria: height of epithelium and number of mitoses. In this paper however the response is considered as a whole without further differentiation. A response is positive whenever the protoplasmic margin exceeds one-third of the cell height or when at least one mitosis is discovered per visual field at a moderate magnification.



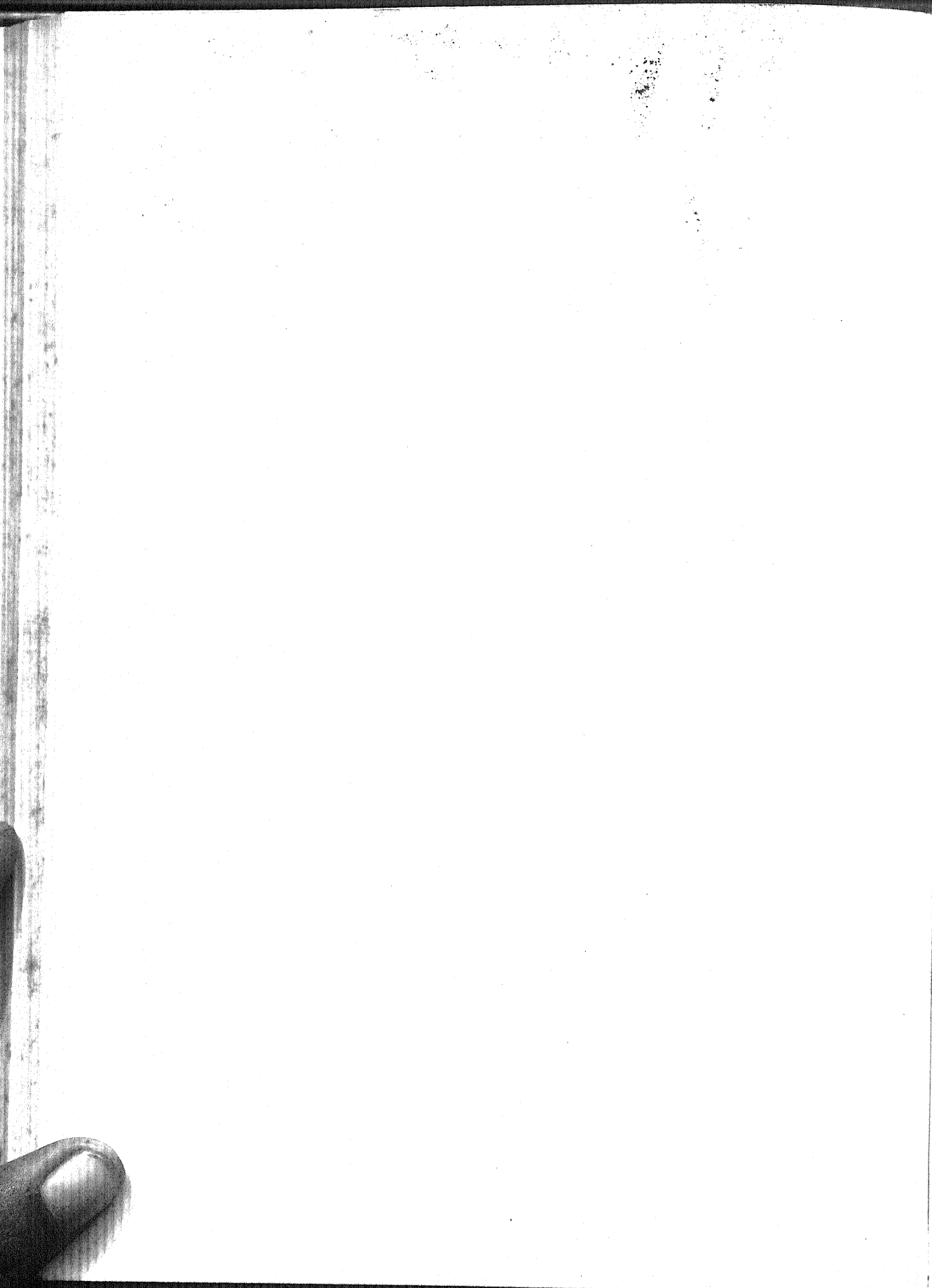
The photomicrographs represent seminal vesicles of infantile castrated male rats. 1, 2 and 3 all at the same low magnification.

Fig. 1. Without treatment; atrophic smooth muscle and epithelium.

Fig. 2. Treatment with menformon; hypertrophic smooth muscle, atrophic epithelium.

Fig. 3. Treatment with male hormone combined with menformon; totally hypertrophic seminal vesicle with ramified lumen and secretion.

Fig. 4. The same preparation as Fig. 3 at higher magnification, showing details of hypertrophic epithelium (note mitoses).



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In Table II 940 animals are classified according to weight of seminal vesicles and positive or negative response of the epithelium. From this we see that epithelial responses become positive between 16 and 19 mg., while above 20 mg. with one exception, no negative response occurs. Positive responses below 14 mg. are exceptional. From this table it appears reasonable to assume 19 mg. as the *positivity limit*.

Table II.

Weight of seminal vesicles in mg.	Epithelial response		Percentage		Number of animals
	Positive	Negative	Positive	Negative	
4-5	0	16	0	100	16
6-7	0	36	0	100	36
8-9	1	68	0.15	99.85	69
10-11	3	109	2.7	97.3	112
12-13	9	125	6.7	93.3	134
14-15	26	121	17	83	147
16	13	56			
17	21 34	38 94	27	73	128
18	28	17			
19	32 60	3 20	75	25	80
20-21	69	0	100	0	69
22-23	37	1	98	2	38
24-25	27	0	100	0	27
26-27	17	0	100	0	17
28-29	14	0	100	0	14
30-31	7	0	100	0	7
32-33	4	0	100	0	4
34-35	4	0	100	0	4
36-37	4	0	100	0	4
38-39	3	0	100	0	3
40 and more	31	0	100	0	31
Total	350	590	—	—	940

Sixty-four animals are not classified in Table II, because the treatment of these marks them as a special group. Their epithelial response was consistently negative, although the weights of their seminal vesicles were between 14 and 30 mg. They were treated with one of the following kinds of preparations: (1) unpurified or crystalline oestrin (menformon); (2) crystalline menformon *plus* subthreshold doses of male hormone; (3) oestrin fractions of male urine after separation from male hormone.

Table III shows the distribution of the cases according to the weight of the seminal vesicles.

Table III.

Weight of the seminal vesicles in mg.	Number of (negative) epithelial responses
14-15	1
16-17	4
18-19	28
20-21	13
22-23	9
24-25	6
26-27	2
28-29	0
30	1
Total	64

From Tables II and III, the following conclusion seems justified. In low doses male hormone is inert. In comparatively high doses, in seminal vesicles of above 14 mg. weight, the frequency of "positive" epithelial responses increases

rapidly until above 19 mg. practically all the reactions are positive. However, there are doses of male hormone insufficient to produce epithelial changes but just sufficient to help oestrin in producing muscular hypertrophy. Hence in seminal vesicles between 14–30 mg. histological control is advisable to detect whether the effect is merely due to oestrin and subthreshold doses of male hormone, or whether the dose of male hormone was sufficient to cause epithelial hypertrophy. Of course not all animals respond to threshold doses.

Reliability of the reaction.

Three criteria are decisive with reference to this question.

(1) *Individual response variations within one group of experimental animals.* This criterion is in favour of reliability in a large majority of the seminal vesicle reactions.

(2) *Increasing response to increasing doses.* Only those experiments are to be used for comparison which were carried out at the same time, so as to exclude group variations due to circumstances changing with time. 126 serial experiments with two or more doses and with exclusion of all those cases where all doses were subthreshold yielded the following result:

	Times	Percentage
Higher dose—increased response	87	69.05
Different doses—equal response	24	19.05
Paradoxical result, higher dose, weaker response	15	11.9

The doses varied as 1:2:4:8 and so on. A group response of otherwise comparable animals was considered different when weight differences of the seminal vesicles averaged more than 2–3 mg., or when histology gave the impression of higher intensity of response.

(3) *Reproducibility of results.* This criterion includes the time factor of response. 57 times threshold doses of the same fractions of a preparation were administered repeatedly to groups of rats during different weeks. 21 experiments were discrepant, 36 yielded identical results. Of these latter however both experiments were negative 16 times and so of doubtful value in this connection. Our last criterion is thus unsatisfied, at least as long as the exact amounts of oestrin contaminating our preparations are unknown.

Influence of body weight and growth.

In Table IV the influence of body weight upon the growth of seminal vesicles is indicated. This table is constructed as follows: 3 animals of one experiment are classified as "light," "medium" and "heavy" in the corresponding horizontal

Table IV.

Weight of animals	Weight of seminal vesicles			
	Light	Medium	Heavy	
Light	235	95	107	Total: 1189
Medium	109	109	88	
Heavy	107	75	264	

lines of the table, in the columns according to the weight of their respective seminal vesicles. Every animal is placed by comparison with the two others of the same experiment. When two animals of a group have equal weights, they are placed in the "light" and "heavy" rows in the columns corresponding to their respective seminal vesicles.

Table IV refers to 1189 animals, treated with potent preparations, showing a marked tendency to respond better with increasing weight. By considering the diagonal of this table from left above to right below as the median line of normal reactions and taking the sum of the figures along it and along the lines parallel with it, the following frequency range results: 107, 184, 608, 183, 107. This is a normal or a Gauss frequency curve, in which the summit is along the diagonal connecting those values which show that larger animals tend to react more strongly than small ones.

Similar conclusions may be reached by considering the influence of growth of the animal during the experiment upon growth of the seminal vesicles. This is shown in Table V.

Table V.

Growth of animals	Weight of seminal vesicles			
	Light	Medium	Heavy	
Small	221	84	148	Total: 1161
Medium	81	81	84	
Great	143	99	220	

Frequency distribution along diagonals: 143, 180, 522, 168, 148.

From these figures we see a tendency for the seminal vesicles to grow most in animals which themselves grow most.

The conditions for optimum growth of seminal vesicles are present in comparatively large and actively growing animals. By introducing body growth as essentially affecting seminal vesicle response to treatment, the existence of a number of external and internal factors is revealed in these experiments. These again account for the extraordinary variability of the reaction and for the difficulty of reproducing threshold reactions at different times. The influence of hypophyseal (growth?) factors upon this reaction will be discussed in another paper. The necessity of a large number of experiments preceding any kind of consistent conclusion is explained by these circumstances.

PART II.

This part deals with the experimental determination of the rôle of the follicular hormone (oestrin) in the development of seminal vesicles.

Varying potency of capon units of male hormone in rats.

Forty-one preparations and fractions of male hormone, extracted from testicles and from male urine, were tested on capons and on rats. In Table VI the comparative values of potency in these two kinds of animals are noted. Standardisation could not be done completely in all cases, the probable minimum active quantity is in many cases more or less than the one noted in the table, both in rats and in capons. Yet from the figures it is apparent that a more exact determination would have made the irregularity of response of rats in most cases still more marked.

A large number of preparations, obtained by the same procedures as those noted in Table VI, were tested on rats only. While the quantity of male hormone in such preparations, whether obtained from urine or from testes, when determined by the capon test, became with increasing experience almost constant,

Table VI.

Prep. from testis	g. tissue con- taining 1 capon unit	g. tissue con- taining 1 rat unit	1 capon unit equal to rat units	Prep. from urine	Litres urine con- taining 1 capon unit	Litres urine con- taining 1 rat unit	1 capon unit equal to rat units
107	100	100	1.0	120 (II)	2.0	0.4	<0.2
112	20	10	0.5	139 (I)	0.5	>0.5	>1.0
113	50	150	3.0	149 (I)	0.5	0.8	1.6
114	100	>20	>0.25	178 (I)	0.5	0.4	?1.0
131	80	20	0.2	178 (II)	5.0	>1.0	?0.2
141	50	15	0.3	294 (II)	1.0	>1.0	>1.0
143	50	20	0.4	315	2.0	2.0	1.0
147	25	40	1.6	315 (II)	2.0	>4.0	>2.0
167	<?50	50	>1.0	Z 12	<0.25	<2.0	?0.1
273	50	>50	>1.0	Z 15	2.0	0.1	0.05
Z 4	50	25	0.5	Z 16 (I)	1.0	>0.4	>0.4
Z 7	25	25	>1.0	Z 22	0.2	0.2	1.0
Z 13	25	>40	>1.6	Z 26 (I)	3.0	≈ 0.4	0.14
338	50	80	1.6	Z 26 (II)	3.0	>0.4	>0.14
358	50	>50	>1.0	390	2.0	>0.4	>0.2
Ho	50	80	1.6	390 (II)	2.0	>0.4	≈ 0.2
470	100	≈ 100	>1.0	390 (II)	2.0	>0.4	≈ ?0.2
491	50	>50	>1.0	455	0.5	>0.4	>0.8
510	50	>50	>1.0	455 (II)	0.5	0.2	>0.4
				459	0.5	>0.2	>0.4
				459 (II)	0.5	>0.2	>0.4
				514	0.5	0.8	?1.6

the potency of such preparations in rats remained very variable; hence the chemical procedure applied for the preparation of male hormone active in birds must be considered inadequate for obtaining a product, which is fully active in rats. (According to recent results, by improved methods the number of bird units found in testes is about 60 units per kg.; in urine about 30 units per litre.)

Pace-making.

In previous sections the importance of ketohydroxyoestrin for seminal vesicles has been emphasised. Besides this form of oestrogenic material, other substances have been described by Marrian [1930], Butenandt and Marrian [1931], Doisy and Thayer [1931], Girard *et al.* [1932], de Jongh *et al.* [1931] and others, which are chemically related to the original follicular hormone (menformon), but have different oestrogenic potencies. The procedure of purification of male hormone includes the elimination of these substances as well as of menformon. Quantitative data with reference to the biological potency of these substances are scanty, owing to their incompletely defined biological effects. The experience gathered in experiments such as are noted in Table VI led to systematic study of the influence of elimination of oestrogenic material and homologues from purified male hormone preparations. It was ascertained that their loss of potency was related somehow to their freedom from oestrogenic material. Preparations, which as crude extracts were potent both in rats and in capons, became inactive in rats in both fractions after separation of the male from the follicular hormone, while the fraction containing the male substance when tested in capons, proved to be almost as potent as the crude extract. This observation led us to the attempt to recover the original activity in rats by recombining the two isolated fractions, after some purification of both in the separated state. This procedure was successful. In Table VII an example is given. The fraction used was the same as referred to already in Table VI under Z 26.

Table VII.

Fraction of urine extract	Daily dose in litres of urine	mg. seminal vesicles found in rat			Histology of epithelium
		I	II	III	
I (male hormone)	0.1	9	9	—	—
	0.2	10	10	—	Negative
	0.4	12	7	9	—
II (follicular hormone)	0.1	14	11	14	—
	0.2	11	16	—	Negative
	0.4	—	19	16	—
III (I + II)	0.1	23	13	—	—
	0.2	24	20	—	Negative
	0.4	27	25	—	—

From this table we see a considerable weight increase of the seminal vesicles after recombining the two fractions of the same preparation. The epithelium of these organs was "negative," while the smooth muscular tissue of group III in all cases yielded the picture of an intensive menformon effect. From Table VI it can be seen that Z 26 was a very weak preparation as far as male hormone is concerned and compared with other extracts of urine. (In Table VI the preparations marked with II are oestrin fractions, these being almost inactive in capons.) Apparently a small amount of male hormone, insufficient to affect the epithelium, may reinforce considerably the effect of oestrin upon the smooth muscle tissue of the seminal vesicles.

In Table VIII a case of combination of male hormone extracted from testicles with a menformon fraction of urinary extract is presented. (The latter is the same as in Table VII.)

Table VIII. *Preparation 338 and Z 26 (II).*

Capon units daily of 338	Urine litres equivalent daily of Z 26 (II)	mg. seminal vesicle found in rat		
		I	II	III
0.2	—	17	9	7
0.4	—	17	14	12
0.8	—	14	17	10
0.2	0.1	20	25	21
0.4	0.2	24	25	28
0.8	0.4	30	34	40

This experiment was done in July 1931, when we still thought that seminal vesicles weighing more than 19 mg. have always a "positive" epithelium. Later a few of these organs were investigated again. It was found that the one weighing 30 mg. (see Table VIII) also had "negative" epithelium. Again male hormone of relatively low potency reinforced the effect of oestrin upon smooth muscle tissue. The final proof that the effect of male hormone was also benefited by the combination with menformon, was obtained from experiments where male hormone was combined with crystalline menformon. These experiments are presented in Table IX. In this Table, whenever histological investigation was done, the negative (—) or positive (+) epithelial response is noted.

In Table IX only 27 parallel experiments on 3–12 animals each are presented, while 11 preparations of male hormone of varying purity were tested. These preparations were combined with doses of crystalline menformon varying from 6 to 100 units a day. A large number of similar experiments done later confirm these results.

Table IX.

Preparation of male hormone	Capon units daily	Average weight seminal vesicles in mg.	Histol. of majority of cases	Additional menformon M.U. daily	Average weight seminal vesicles in mg.	Histol.
153	0.75	7.8	-	6	7.5	-
167	2.0	29	+	6	24	-
338	0.4	12	.	10	12	-
167	1.0	20	+	15	15	-
514	0.8	12	-	20	11	-
149 I	0.2	7.5	-	30	8	-
167	0.2	11	-	30	16	+
358	0.25	10	-	40	21	-
358	0.5	14	-	40	20	.
338	0.4	10	-	50	17	+
358	0.25	11	-	50	17	-
147	0.4	17	-	60	22	+
358	0.25	11	-	60	28	.
358	0.5	14	-	60	22	.
470	1.0	6	-	60	15	-
470	0.8	6	-	60	13	-
491	1.0	19	.	60	20	+
491	0.75	10	-	60	15	-
455	0.2	10	-	60	15	-
459	0.17	10	-	60	19	+
459	0.2	9	-	60	14	? +
459	0.4	7	-	60	16	+
459	0.8	9	-	60	16	+
459	1.6	17	+	60	26	+
338	0.2	11	-	100	14	.
338	0.4	12	-	100	23.5	+
338	0.8	14	.	100	24	.

Less than 40 M.U. of menformon seem to have no effect when combined with male hormone. 60 units a day regularly reinforce the seminal vesicle reaction (of another 30 similar experiments 23 showed this phenomenon). The number of positive epithelial responses also increases, though less regularly. 0.4 capon unit of male hormone without oestrin hardly ever produces seminal vesicles of more than 14 mg. in 5 days, while 1.6 units a day are a dose, which with some regularity yields appreciable growth of the seminal vesicles without the aid of oestrin. In the majority of cases 0.4 unit, reinforced by 60 units of menformon, is sufficient to produce the weight of seminal vesicles at which positive epithelial response seems to be possible. From these data we may conclude that menformon increases the effect of male hormone about 4 times. A simple addition could not explain this increased reaction, first because menformon has no effect upon the epithelium of seminal vesicles even in much larger doses than those here applied, and further because 60-200 M.U. of menformon alone tested in 24 animals yielded only exceptionally seminal vesicles of more than 11.6 mg. in 5 days.

Pace-making may thus be characterised as follows: our male hormone preparations, having a very much reduced content of menformon (as tested in castrated mice) act together with crystalline menformon upon the smooth muscle tissue of seminal vesicles. The two substances mutually reinforce each other's activity in this respect. Epithelium of seminal vesicles is influenced only by male hormone in a positive sense. This effect seems to depend on many accessory conditions. One of these is a certain dimension of the non-epithelial tissues of the seminal vesicles, seeing that according to our experience the frequency of positive epithelial responses increases rapidly in seminal vesicles of more than

19 mg. weight. The considerable number of cases in which (at least in infantile rats), in spite of comparatively large seminal vesicles, no positive epithelial response occurred proves that the size of seminal vesicles and a certain amount of male hormone are not yet always sufficient for a positive epithelial response, therefore this cannot be considered as a reliable test for male hormone as long as the rôle of other effective agents is not elucidated.

Besides menformon trihydroxyoestrin (Marrian) (crystals of Marrian) was tested in pace-maker experiments. Two preparations of male hormone (338 and 358) were used in these experiments. Their effect, when given alone, may be seen in Table IX. Table X shows the results of these experiments.

Table X.

Preparation of male hormone	Capon units daily	γ -Trihydroxy-oestrin daily	Average weight in mg. of seminal vesicles	Histology
338	0.4	1	14	-
338	0.4	3	12	-
358	0.3	4	15	-
358	0.3	5	17	.
358	0.5	5	22	.
338	0.4	5	15	+
338	0.3	5	11	-
338	0.3	6	17	.
358	0.2	10	20	+
338	0.4	10	18	+
338	0.8	10	21	+
338	0.4	20	15	+
338	0.8	20	18	+

From these data the conclusion seems justified that trihydroxyoestrin, when combined with male hormone, reinforces seminal vesicle response to about the same extent as menformon (ketohydroxyoestrin). A contamination of the preparation of trihydroxyoestrin with menformon could not possibly account for this result, because both preparations were tested on castrated female mice and the oestrogenic potency of the trihydroxy preparation was so weak that 20 γ did not correspond to more than 1.5 γ menformon, *i.e.* about 15 units. This amount of menformon is too little for pace-making. The possibility of contamination of both with a very active third substance still deserves consideration.

For trihydroxyoestrin this is a new biological effect, which if confirmed might serve as a method of testing. The fact that related compounds with comparatively low oestrogenic potency have about the same pace-maker effect as menformon, might perhaps account for comparatively strong responses of seminal vesicles to crude testicular extracts, while the testicle is known to contain very little actual oestrogenic substance. It has hitherto been impossible to determine quantitatively the non-oestrogenic compounds in various organs on account of the already mentioned lack of an adequate biological method for testing them. Trihydroxyoestrin when injected alone has, in doses of 4-10 γ daily, the same effect as similar doses of menformon, *i.e.* the seminal vesicles show a slight muscular hypertrophy, and their weight reaches in 5 days an average of 11.5 mg.

An attempt to analyse pace-making.

The following experiment was done in order to ascertain whether pace-making depends on simultaneous administration of the two hormones involved or not. 30 rats were castrated, 15 of these were treated during 6 days with 60 m.u. of menformon daily, 15 left without treatment. On the 7th day the first group of 15 was divided into three groups of 5, their treatment with

menformon was stopped, they were treated daily, as usual, with 0.2, 0.4 and 0.8 capon unit of male hormone respectively, while the group of 15 animals, which were hitherto without treatment, was also divided into three groups of 5, to which the same doses of male hormone as to the first three groups and additional 60 units of menformon daily were administered. On the 5th day of the second stage of this experiment the animals were killed. The result is shown in Table XI.

Table XI.

Capon units daily	Average weight in mg. of seminal vesicles after treatment	
	In 2 stages	Simultaneously with 2 hormones
0.2	11	15
0.4	12	17
0.8	17	19

Only one of these groups showed histologically positive epithelial response, *viz.* that treated simultaneously with menformon and with the highest dose of male hormone.

A treatment with menformon preceding that with male hormone does not yield the same strength of pace-maker effect as does the simultaneous treatment with both hormones.

Anomalous histological phenomena.

In 58 groups of 3 or more animals treated by mixtures of male and follicular hormones, the seminal vesicles exhibit a partial detachment of their epithelial lining from the wall of the organs. The rounded cells are conglomerated in the centre of the lumen. This result occurred only twice after exclusive treatment with male hormone extracted from testicles; it was observed 56 times in cases where male hormone was combined with crystalline or non-crystalline oestrin and with trihydroxyoestrin. By prolonging combined treatments the regularity of this occurrence is enhanced. The phenomenon hardly ever occurred where a "positive" epithelial response was obtained with male hormone. It is ascribed to an unbalanced influence of oestrin. Similar epithelial changes, not unlike tumours, were observed in rabbits' uteri after a treatment with several thousand units of follicular hormone for a few months. Lately de Jongh in our laboratory (unpublished results) observed not only epithelial degeneration, but also a metaplasia from columnar to stratified epithelium in the proximal part of the seminal vesicles of mice and rats after prolonged treatment with oestrin. This significant result will be extended and published in due course.

DISCUSSION.

An optimum growth of seminal vesicles under artificial conditions is not to be obtained by treatment with one hormone only (*e.g.* the male hormone). The effect of this substance upon smooth muscle tissue is only moderate, its main point of attack being the columnar epithelium. Oestrin and its various homologues, of which only one is mentioned in this paper (trihydroxyoestrin), act upon smooth muscle tissue, while they either do not affect columnar epithelium or induce degeneration. The structures resulting from forced treatment with these substances are somewhat reminiscent of tumours and open a possible development of tumour pathology.

The combined effect of follicular and male hormones upon seminal vesicles is a mutual reinforcement of each other's actions. For this phenomenon the

expression "pace-making" is suggested. Complete restitution of seminal vesicles in animals castrated while they were immature and treated after they became adult was not obtained even by combined treatment. This fact indicates already that compounds should be sought amongst the homologues of oestrin, which are still more potent than the ones used. We surmise that one of the homologues of oestrin might be more potent with reference to smooth muscle hypertrophy than oestrin itself and might account for the higher potency of crude extracts of male hormone than of its purified forms. It is reasonable to assume that natural development of male secondary sex organs is governed by two types (male and follicular) of hormones. It is obvious that for the measurement of the oestrin homologue active towards seminal vesicles, its oestrogenic potency gives no reliable basis. Hence we are unable to say without analysis of the effect on seminal vesicles of castrates, how much of these compounds does occur in testicles. This conception inaugurates a new line of research on "male hormones." It is quite certain that experiments, such as *e.g.* those of Moore [1932], where "complete" restitution was obtained, the preparations used must have contained besides male hormone the active principle which is highly potent in producing muscular hypertrophy. The same applies to other muscular organs, such as the vas deferens, also studied by Moore and by others.

Under these circumstances there is no question of a standardisation of "male hormone" (under this name being understood the substance acting upon the comb of capons) in these experiments. The use of rats for such a purpose though not impossible, could not be recommended before these matters are settled by careful biological analysis.

A further conclusion from these results is that antagonism of sex hormones within the secondary sex organs certainly does not exist, at least between male and female hormones. They do not inhibit each other's effects, but on the contrary, in adequate doses, they support each other (pace-maker effect). The noxious influence of an unbalanced treatment with one of these substances may be alleviated or even turned to advantage by administration in adequate doses of the other one, but in no way is this balancing based upon inhibition.

The therapeutic application of one of these substances may fail without simultaneous application of the other.

The successful application of combined male and female hormone preparations in the same proportion as mentioned above by van Cappellen [1933] in the treatment of prostate hypertrophy confirms the last statement from the side of clinical experience.

SUMMARY.

1. Simultaneous experiments on capons and on infantile male rats showed loss of activity of male hormone after purification as regards rats, while activity was retained as regards capons.
2. Oestrin promotes smooth muscle development in the secondary sex organs of mammals, hence its "paradoxical" positive growth effect with reference to seminal vesicles is only apparent. Male hormone is active in promoting epithelial hypertrophy in the same organs.
3. In hypertrophied seminal vesicles histology decides between these two types of effect.
4. Experiments on infantile rats are reliable as regards individual response in one group of animals, less so with reference to dose-action ratio, while weekly response variations are rather considerable.

5. Body weight and growth during the experiments have a marked effect upon the response to potent preparations.

6. Male and follicular hormones support each other's effects upon seminal vesicles. This is called "pace-making."

7. Trihydroxyoestrin, though its oestrogenic potency is much less than that of ketohydroxyoestrin (menformon), is an effective pace-maker in the same doses (6-10 γ) as menformon. Our knowledge about the quantities of these and homologous substances occurring in organs and tissues is scanty.

8. Abnormal histological responses of the seminal vesicles may occur after treatment of the animals with inadequate combinations of sexual hormones. The significance of these phenomena, reminiscent of tumours, remains unexplained.

9. In clinical practice, the application of these substances is advised on grounds of symptomatology and the knowledge of their pharmacological effects, rather than on so-called "hormone-balance," which does not give any idea about the mutual influence of these substances. Therapeutic application of combined male and female hormone preparations in the treatment of prostate hypertrophy has proved successful.

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CXCVI. ABSORPTION SPECTRA IN RELATION TO THE CONSTITUENTS OF FISH OILS.

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(Received July 14th, 1933.)

IN comparison with other organic materials the vegetable fats and oils exhibit, as a rule, a high degree of transparency to ultra-violet rays. Thus in many instances, *e.g.* olive oil and groundnut oil, a thickness of several millimetres of oil is necessary for appreciable absorption to be shown, and, even so, little of a selective nature is exhibited. From this it follows that the glycerides of the common fatty acids, saturated and unsaturated, are relatively transparent. The same is true of mammalian- and fish-body oils which do not contain vitamin A.

In the case of fish-liver oils the greater part of the selective absorption is usually due to vitamin A, but in a number of cases, *e.g.* codling-liver oils [Lovern *et al.*, 1931] the gross absorption is so low, sometimes only recorded by the use of undiluted oil, that it becomes reasonable to conclude that the absorption shown by the glycerides of the polyethylenic acids occurring in nature is inconsiderable and exhibits but little selectivity.

In these circumstances it is somewhat surprising to find that some constituents of the total acids obtained by saponification of many fats, particularly fish-liver oils, exhibit relatively intense absorption of a highly selective nature, a long series of sharply defined narrow bands, about $10m\mu$ wide, being regularly observed, together with a few inflexions and fainter bands which are not always seen. After allowing for the vitamin A content of the richer oils it is found that the "acid" curve invariably lies well above the "oil" curve, showing the absorption to be not only more selective, but also considerably more intense, than that of the glycerides and other esters in the oils.

Table I summarises the spectral absorption of the total acids from a number of fish-liver and fish-body oils. The last three in the table are interesting in that, although they are not liver oils, they contain appreciable quantities of vitamin A.

Before attempting to interpret the above data it is essential to recall the main facts concerning the spectral absorption of those constituents of natural fats which do not belong to the fatty acids, namely glycerol, cholesterol, ergosterol, vitamin A, vitamin D, certain higher alcohols, *e.g.* oleyl, batyl, selachyl, chimyl and in certain cases (shark, dog-fish) the hydrocarbon squalene, which, however, shows no selective absorption and may be dismissed here. Many oils also contain small quantities of coloured substances not unlike carotenoids [*cf.* Lovren and Morton, 1931]. Glycerol, although nearly always the predominant non-acid constituent, amounting to 4-5 % of the original oil, does not absorb

Table I. *Absorption maxima of total acids from saponification of fish oils.*

Maxima and inflexions (italics) are given in $m\mu$, with the $E_{1\text{ cm.}}^{1\%}$ values immediately beneath. Thus $E_{1\text{ cm.}}^{1\%}$ 0.12 implies an absorption band with a maximum at $378 m\mu$, and an $E_{1\text{ cm.}}^{1\%}$ value (i.e. $\log I_0/I$ for a 1 cm. layer of 1 % solution) of 0.12.

Monk (angler-fish)-liver oil	—	(420 400 377)	347 328 315.5 301	—	281 269 260 234 $m\mu$
		(0.2 0.3 0.4)	1.5 2.0 9.0 10.0		68 82 74 116 $E_{1\text{ cm.}}^{1\%}$
Haddock-liver oil	—	445 423 400 377 362	350 330 316.5 301	—	282 269 — Tail-off
		0.032 0.038 0.052 0.075 0.13	0.225 0.285 0.63 0.72		1.32 1.55 — 22
Turbot-liver oil	460	442 402 378 360	350 333 317 301	288	282 270 — Tail-off
	0.07	0.1 0.14 0.21 0.31 0.45	0.7 0.9 1.18 1.45	2.3	2.72 2.76 — 26
Pollack-liver oil	460	422 401 377.5 360	349 331 316.5 302	290	282 270 — Tail-off
	0.017	0.027 0.055 0.1 0.11	0.22 0.27 0.55 0.67	1.1	2.4 2.7 — 28
Ling-liver oil	—	422 402 377 360	349 332 316.5 301	294	283 270 — Tail-off
		0.1 0.14 0.2 0.25	0.42 0.55 0.76 0.97	1.4	2.2 2.3 — 24-25
Dab-liver oil	—	423 402 378 360	348 333 316.5 302	290-5	282 270 — Tail-off
		0.02 0.07 0.12 0.16	0.28 0.72 0.97 1.2	1.7	2.36 2.4 — 18
Cod-liver oil	—	— 392 375 360	350 330 316 302	—	281 270 259 234
		1 1.5 2 14	15 80 85		210 250 205 200
Sturgeon peritoneal fat	—	— 400 377 361	348 331 316 301.5	—	282 269 259 Tail-off
		0.025 0.05 0.065 0.15	0.2 0.31 0.35		0.93 1.2 1.25 22-23
Sturgeon pancreatic fat	—	— 400 377 359	348 332 317 300	—	282 270 259 Tail-off
		0.042 0.08 0.12 0.21	0.3 0.55 0.65		1.1 1.14 1.1 18-20
Conger eel peritoneal fat	—	423 401 377 361	349 332 316.5 302	—	283 270.5 — Tail-off
		0.12 0.19 0.5 0.65	1.0 1.1 1.4 1.42		2.9 3.1 — 20

selectively over the region concerned. The remainder, known collectively as the "unsaponifiable matter," usually accounts for about 1-1.5 %¹ of the original oil, and (in an average cod-liver oil) has approximately the composition:

Cholesterol and higher alcohols	...	About 96 %
Vitamin A	...	3-4 %
Ergosterol	...	(Order) 0.1 %
Vitamin D	...	0.02-0.03 %

Cholesterol is practically diatinctic; a 10 cm. layer of 4 % solution shows no selective absorption and only very little general absorption in the extreme ultra-violet. Similar considerations apply to the higher alcohols, as would be expected from their saturation; in fact neither cholesterol nor the higher alcohols can be detected spectroscopically in natural oils. The remaining three substances all show strong selective absorption.

Vitamin A, which is responsible for most of the absorption of liver oils in the near ultra-violet, has a characteristic broad band with a maximum at $328 m\mu$. A portion of the observed intensity at $328 m\mu$ is not due to vitamin A, as is shown by discrepancies between the vitamin A potency calculated from this value and from feeding tests and antimony trichloride blue values. However, since these discrepancies largely disappear when the unsaponifiable matter instead of the oil itself is used in the estimation [Coward *et al.*, 1931] it would seem that the interfering absorption is due to certain of the acid constituents, which are considered later. On the basis of the values: $E_{1\text{ cm.}}^{1\%}$ $617 m\mu$ 5000, $580 m\mu$ 2600, and $328 m\mu$ 1600, Carr-Price, 78,000, for apparently pure vitamin A [Carr and Jewell, 1933] an average good cod-liver oil contains about 0.04 % of vitamin A. The figure varies considerably from fish to fish and even more so from species to species, the range covered within our experience by mature fish

¹ This applies to the liver oil from most fishes, including the average cod, but certain fish-liver oils are so rich in one constituent (e.g. vitamin A in halibut; squalene in elasmobranch fishes) as to increase this figure considerably, up to 10 % or more in special cases.

being from 0.004 % in haddock-liver oil to about 10 % in a sample of halibut-liver oil representing the mixed oil from several livers. No typical value can therefore be given. We have considered these variations in a separate paper [Lovern *et al.*, 1933].

Ergosterol exhibits intense, highly selective absorption in the middle ultra-violet, with maxima at 293.5, 281.5, 270.6, 260, 250.3, 242 $m\mu$, $E_{1\text{cm.}}^{1\%}$ max. being of the order 290 (in alcohol). Thus with 0.001 % of ergosterol (a high figure for a fish oil) the absorption intensity ($\log I_0/I$) in this region for a 1 cm. layer of a 1 % solution of the oil would be about 0.003, an obviously insignificant contribution to the u.v. absorption at 280 $m\mu$ usually observed, which ranges from 0.4 upwards in the typical fish-liver oils examined. This explains the observation of Morton *et al.* [1931] that the intensity of absorption in this region cannot be correlated with the sterol content.

Vitamin D (calciferol) in the pure state also absorbs strongly in the middle ultra-violet, having a single broad band, free from fine structure, with a maximum at 265 $m\mu$ ($E_{1\text{cm.}}^{1\%}$, 265 $m\mu$, 485). Askew *et al.* [1932] found a mean anti-rachitic value of 40,000 International Units per mg. of purified calciferol; typical cod-liver oils range from 25 to 200 units per g. Hence the vitamin D content of a cod-liver oil is, on this basis, of the order 0.00006–0.0005 %, giving for a solution of the oil an $E_{1\text{cm.}}^{1\%}$ value of 0.0003–0.0025, which obviously cannot influence the measured absorption curve of the oil. Even in puffer fish-liver oil, which it is stated [Bills, 1927] may be 15 times as rich in vitamin D as cod-liver oil, the effect would be inconsiderable.

Vitamin A, then, is the only non-acid material contributing appreciably to the ultra-violet absorption of most fish-liver oils. This, of course, is to be expected from the facts that it is both intrinsically more strongly absorbing than either ergosterol or vitamin D, and is also usually present in vastly greater proportions.

Upwards of a dozen different fatty acids combined as triglycerides are present in most fish-liver oils, a feature of which is their high content of unsaturated acids with 20–22 carbon atoms and four or more double bonds in the molecule. Though the proportions vary somewhat, saturated acids account on the average for 15–20 % of the total weight, mildly unsaturated (1–2 double bonds) about 70 %, and the highly unsaturated, including the so-called clupanodonic acids, 12–15 %. The precise composition of the more unsaturated fractions remains indeterminate by ordinary methods of analysis, in the sense that only the average degree of unsaturation is ascertained (expressed in terms of number of H atoms lacking as compared with the corresponding saturated acid) and not the individual unsaturation of the various members. Thus, to take a simple case, $(-2nH)$ might imply one acid with n double bonds, or an equimolecular mixture of acids with $n-1$ and $n+1$ double bonds. In addition, the extent of conjugation of these double bonds is often a matter of uncertainty, particularly in the more unsaturated members. The latter, incidentally, have also been detected in several different kinds of algae, though in much lower proportions than in fish oils [Tsujimoto, 1925], and in the fat of birds which feed on fish, but not in birds which feed exclusively on insects or grain [Grün and Halden, 1929]. They are thus apparently connected in some way with aquatic life.

Turning now to spectral characteristics, oils which are known to consist mainly of saturated glycerides show either no selective absorption or at most only feeble indications of a group of three bands near 270 $m\mu$. Oleic acid, with one double bond, shows traces of the 270 $m\mu$ group which become fainter as the acid is purified. Linseed oil, which largely consists of the glycerides of

linoleic acid (order 50 %, F_2) and linolenic and *isolinolenic* acids (order 30–35 %, F_3) has an $E_{1\text{cm}}^{1\%}$, 270 $m\mu$ value of 29, which is a mere fraction of that shown in this region by some fish-liver oil acids (*cf.* cod, monk) containing, as is usual, a rather smaller proportion of di- and tri-ethylenic acids than do the acids from linseed oil. It is therefore impossible to associate straight chain di- and tri-ethylenic acids with the highly selective absorption regularly observed at 270 $m\mu$, and we are forced to the conclusion that the acid responsible for this group, if indeed it is a straight chain acid, must have more than three double bonds. This is not inconsistent with other considerations to be mentioned later, which further indicate that the remaining narrow bands exhibited nearer the visible region of the fish-liver oil acids must be associated with a still higher degree of unsaturation.

The C_{20} – C_{22} clupanodonic and higher unsaturated acids remain to be considered as possible causes of selective absorption. Here the mean unsaturation as determined by hydrogenation and iodine values is of the order F_{4-5} , but little appears to be definitely known concerning the extent of conjugation of these double bonds, without which little absorption of a selective nature is to be expected, in the absence of ketonic or other absorbing groups. According to Tsujimoto [1928] there is no conjugation of double bonds in a C_{20} acid which he terms clupanodonic, and which is known to possess a straight chain of carbon atoms from its conversion into behenic acid by hydrogenation; but the term "clupanodonic" is used somewhat loosely in the literature, sometimes being confined to the C_{20} and C_{22} acids with 4 or 5 double bonds, and sometimes including everything from C_{20-22} upwards¹. The highly absorbing acids are evidently to be found somewhere among, or else are derived from, the polyethylenic higher acids. There are three possibilities: (i) that they are the "clupanodonic" *etc.* acids themselves; (ii) that they belong to a series of as yet unidentified compounds which, although perhaps very highly unsaturated, are present in such small quantities that they contribute little to the general unsaturation; (iii) that they are derived from the "clupanodonic" acids by isomerisation, cyclisation or other chemical change. In case (i), even if in some of the acids all the double bonds were conjugated, one would expect a series of relatively broad absorption bands, one from each acid, superposed to form an ill-defined summation curve, rather than the sharply defined narrow bands actually observed. Further, since the typical fish-liver oil of low free fatty acid content but rich in clupanodonic glycerides—using the term "clupanodonic" in its widest sense—is devoid of narrow bands, and since synthetic glycerides prepared from the highly absorbing total acids, including clupanodonic, *etc.*, exhibit the same fine structure as these acids, though at a slightly lower intensity [Gillam *et al.*, 1931] the interesting position arises that either the clupanodonic acids are not responsible for any of the observed fine structure, or the configuration of the acidic portion of the natural clupanodonic glycerides or esters differs from that of the clupanodonic acids themselves as prepared by thorough saponification of these glycerides [Morton *et al.*, 1931]. The same thing applies, of course, to any even more highly unsaturated acids which might be present.

Against case (ii) is the extraordinarily high extinction coefficients which must be postulated for the highly absorbing acids if they are present only in very small quantities.

Case (iii) provides the best working hypothesis, although it is not yet known with certainty whether the change in configuration (which appears to be irreversible) occurs during the saponification process or during the subsequent

¹ Inone and Sahashi [1932] give a $C_{22}F_5$ acid as containing an acetylenic linkage.

acidification. The evidence available points strongly to the former alternative and is supported by the recent work of Dann and Moore [1933], the results of these authors suggesting the existence of some connection between the length of time during which the alcoholic KOH solution is boiled (usually much longer than is strictly necessary for saponification) and the quantity of highly absorbing acids produced.

The possibility of an isomeric change in the acidic portion of certain glycerides giving rise to the narrow bands may account for a phenomenon which has been observed on more than one occasion. Sturgeon peritoneal fat containing an appreciable amount of vitamin A ($E_{1\text{ cm.}}^{1\%}$ 602 $m\mu$, 0.34; 568 $m\mu$, 0.3) when first examined showed only two bands in the ultra-violet, one at 328 $m\mu$ $E_{1\text{ cm.}}^{1\%}$ 0.28, and one at 281 $m\mu$, $E_{1\text{ cm.}}^{1\%}$ 0.26, with no detectable fine structure. On re-examination some 4 months later bands were found at 460, 399 and 377 (inflexions), 325, 292, 282, 271 $m\mu$, the 325-330 $m\mu$ intensity remaining unchanged within experimental error ($E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$, 0.27) while the rest of the bands ranged from $E_{1\text{ cm.}}^{1\%}$ 0.0065 (460 $m\mu$) to 0.54 (271 $m\mu$). Similarly, a fresh solution of ling-liver oil (5 %) in heptane exhibited a broad band at 321 $m\mu$ ($E_{1\text{ cm.}}^{1\%}$ 0.5) and very faint traces of selective absorption at 398, 282 and 270 $m\mu$. Two days later, unmistakable signs of selective absorption at 400, 377, 360, 348, 315, 282 and 270 $m\mu$ were observable, together with a general increase in in-

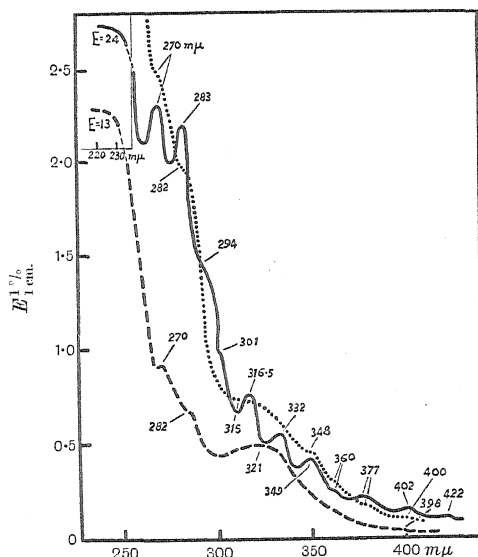


Fig. 1. Ling (*Molva vulgaris*). Solvent: heptane. SbCl_3 blue value of oil: $E_{1\text{ cm.}}^{1\%}$ 603 $m\mu$, 0.54; 578 $m\mu$, 0.44.

— — — Liver oil, fresh solution.
 Liver oil, same solution two days later.
 - - - - - Acids from saponification of liver oil.

tensity of absorption throughout (see Fig. 1). The new curve could in fact be accounted for by superposition of a low intensity ling-liver oil acid curve upon the original ling-liver oil curve.

Two interpretations are possible: either spontaneous hydrolysis takes place, or the fine structure-producing change can occur, without hydrolysis, in the

acidic portion of certain glycerides. It is probable that most of the fine structure observed in stale fish oils is due to one or both of these causes. Which cause predominates can be decided by washing with sodium carbonate and re-examining spectroscopically. That the change can occur within the glyceride molecule as a result of some process other than hydrolysis is proved by the fact that the narrow bands exhibited by a skate-liver oil were not eliminated as a result of washing with sodium carbonate. Further, the experience with ling-liver oil suggests that this non-hydrolytic change is favoured by dilution with a solvent, since the oil itself, which had only just started to show selective absorption apart from the vitamin A band, was by no means fresh.

It has not been possible to trace any parallelism between the vitamin A potency of an oil (as measured by the SbCl_3 colour test) and the intensity of absorption exhibited by the total acid fraction. Moreover, with increasing vitamin content the absence of such correlation becomes increasingly evident. It is therefore inferred that the fine structure acids are not decomposition products of vitamin A, a conclusion already arrived at by Morton *et al.* [1931] and supported by the appearance of narrow bands in the above-mentioned sturgeon peritoneal fat without sensible loss of intensity at $328m\mu$. There is likewise no evidence to connect the acids in any way with vitamin D, and no direct connection has been traced between the acids and the sterol content [Morton *et al.*, 1931]. That the acids are directly associated with the synthesis of either vitamin A or D seems equally improbable for the same reasons, and also because a really fresh fish oil shows very little selective absorption apart from the $328m\mu$ band, but the possibility of their being indirectly connected in some way with vitamin synthesis (*e.g.* as degradation or by-products of intermediate stages in the conversion of carotene) cannot at this stage be definitely excluded. Two significant facts stand out: first that liver oils are an incomparably better source of the highly absorbing acids than vegetable oils (*e.g.* olive, cottonseed); second that, except when the diet contains minimum quantities of carotene, the conversion of carotene into vitamin A is a very wasteful process. It has not yet been explained what happens to the "wasted" carotene.

It will be recalled that the typical total acid absorption curve exhibits the following maxima: $230\text{--}235m\mu$ (always at a high intensity, sometimes a genuine maximum, more often a tail-off), (259), 270, 282, $288\text{--}295$ (inflexion), 301.5, 316.5, 332, 348, 360 (inflexion), 377, 401 (422, 442, 460 inflexions or very weak bands), the figures in brackets being not always strictly reproducible. The question arises whether the whole of the fine structure is due to one acid or whether it is the result of superposition of a number of absorption curves, each arising from a different acid.

Evidence adduced from various sources all points to the conclusion that there are several absorbing entities. This evidence is summarised in Table II, in which the bands are segregated into discrete groups as far as the data warrant. Two different lines of attack have been followed, one based on an examination of the intensities of the various bands, to see whether there is any tendency for certain groups of bands to vary together in intensity, and the other based on frequency differences. The intensity method was applied to (i) the methyl esters of the more unsaturated cod-liver oil acids in which a partial separation had been effected by high vacuum distillation; details of this have already been published [Gillam *et al.*, 1931]; (ii) monk (angler-fish)-liver oil acids, which were partially separated (a) by adsorption on fibrous alumina, (b) by the Twitchell lead salt process, and (c) by distillation of the esters; (iii) the total acids in Table I. Incidentally, the separation of the monk acids by fibrous alumina

Table II. *Grouping of the maxima exhibited by the highly selectively absorbing acids.*

Former work* Fractionation of cod-liver oil acids (from intensity of maxima shown by different fractions)	Present work			
	Fractionation of monk- liver oil acids (from intensity of maxima shown by different fractions)	Acids from several fish-liver and body oils. Theoretical considerations of		
		Intensity of maxima	Frequency differences	
234 $m\mu$	234 $m\mu$	230-5 $m\mu$	230-5 $m\mu$	
259	260	(259)	259	
270	270	270	270	
281	281	282	282	
		290 (inflexion)	295	288
(301)	301	301.5	301.5	301.5
(316)	316.5	316.5	316.5	316.5
	(331)	332	333	332
	(349)	348		348
	—	360 (infl.)		365.5
	375	377		357
	394	401		377
				401
				427

 $\Delta \text{ cm.}^{-1} = 1580, 1570, 1380, 1590$ * Gillam *et al.* [1931].

gave the highest all round values yet recorded for the intensities of the narrow bands $E_{1 \text{ cm.}}^{1\%}$, 269 $m\mu$, 380; 234 $m\mu$, 440.

When a broad absorption band can be resolved into a number of narrow bands, the separation (in wave-numbers) of the components is often nearly constant, the $\Delta \text{ cm.}^{-1}$ value corresponding with a vibrational frequency. The provisional grouping of the maxima shown by our acids can be tested by applying this criterion. Thus if the 270 and 282 $m\mu$ bands are physically related ($\Delta \text{ cm.}^{-1}$, 1580), bands would also be expected at 259 and 295 $m\mu$; and similarly for the other groups in Table II. The figures in heavy type represent the dominant regularly observed bands, while the others are the calculated wavelengths of the fainter bands which one would expect to be associated with the main bands. This particular segregation into groups agrees very well with the main facts and provides a plausible explanation of a number of otherwise apparently anomalous features.

(i) The 259 $m\mu$ band is not always observed. This is to be expected if it is both intrinsically faint and occurs on a steep part of the curve due to the 230-5 $m\mu$ substance, by which it is likely to be masked or at most reduced to a faint inflexion. It should perhaps be explained that a weak band superposed on a steep absorption curve due to another substance appears as an inflexion on that curve.

(ii) A long inflexion, varying in position, is often found about 290 $m\mu$. This can be attributed to the joint effects of the 288 and 295 $m\mu$ faint bands, the middle of the inflexion being nearer to 288 or 295 $m\mu$ according to which substance predominates.

(iii) The overlapping of the two middle groups similarly explains variations of 0.5-1.0 $m\mu$ in the position of the 316.5 and 332 $m\mu$ bands according to the source of the oil from which the total acids were derived.

(iv) The 360 $m\mu$ inflexion is apparently due to the superposition of the two faint 365.5 and 357 $m\mu$ bands, and, forming a link between two sets of bands, accounts for the tendency to group them together as one when intensity data only are considered.

(v) By calculation, a band should appear at $427m\mu$. In practice, one is observed, either as an inflexion or as a weak band, near $423m\mu$. This may be due to interference by a trace of something analogous to the monk pigment [Lovern and Morton, 1931] one of the absorption bands of which lies at $422m\mu$. It should be remembered that the intensity of this part of the "total acid" absorption curve is very low and the curve correspondingly more susceptible to such influences.

There are thus probably at least five highly absorbing entities contributing to the complex absorption curve of the total acids. At this stage we can only speculate as to their inter-relationship and structure, but there are certain general conclusions which can be regarded as not improbable. In common with the other acid constituents, they all appear to be monobasic. This is suggested by the behaviour of the total unsaturated acids on esterification with methyl alcohol and sulphuric acid. In general, monobasic acids esterify readily and almost completely (95-98 %), while dibasic acids show a tendency to stop short at one carboxyl group (*i.e.* 50 %). The acids from fish-liver oils esterify with the same ease and to about the same extent as normal monobasic acids, showing that in the main, at least, the acids are monobasic. Further, the small "un-esterified" portion, in which any partially esterified dibasic acids would collect if present, shows no increase in unsaturation or absorption, thus indicating that the fine structure acids themselves also are monobasic.

Concerning the absolute quantities of the highly absorbing acids present, little can be said at the moment. The percentage of so-called highly unsaturated acids, C_{20-22} , with four or more ethenoid linkages, shows no correspondence with the observed range of $E_{1\text{ cm}}^{1\%}$ values. Thus the $270m\mu$ group of bands has usually an $E_{1\text{ cm}}^{1\%}$ value of 2 or 3 in the acids from the liver oils of most of the species studied, and yet in certain cod-liver oil acids, with about a normal percentage of C_{20-22} , f_{4-5} upwards, the E value was 250. Since the cod-liver oil acids evidently contained 100 times the normal amount of these fine structure acids, and this was not reflected in a measurable increase in the general unsaturation of the polyethylenic fraction, the inference is either that the fine structure acids are present only in minute quantities, or that the change from relatively diactinic unsaturated acids to highly absorbing unsaturated acids leaves the iodine value practically unchanged.

The monk-liver oil acids, which were examined in considerable detail, support these general conclusions. Their approximate composition is as follows:

<i>Saturated acids:</i>			%
Myristic...	4.9
Palmitic...	9.6
Stearic	1.3
<i>Unsaturated acids:</i>			
Myristoleic	0.4 (-2.0 H)
Palmitoleic	12.1 (-2.0 H)
C_{18} group	30.9 (-3.3 H)
C_{20} group	24.9 (-5.9 H)
C_{22} group	15.9 (-8.6 H)

They thus fall in line with the fatty acids from the majority of marine fish-liver oils as regards general composition, but are unusually interesting in that they are associated with a red pigment in the liver oil [Lovern and Morton, 1931] and are from 10 to 30 times as highly absorbing in the ultra-violet as the usual

total acids from most fish-liver oils. Like the cod-liver acids, their high absorption is not reflected in the measured unsaturation, and they give no colour reaction with antimony trichloride. Distillation of the methyl esters under 0.1 mm. pressure results in a very imperfect separation of the highly absorbing constituents, but there is a marked tendency for them to accumulate in the high-boiling fractions and in the residues, a fact which suggests a high molecular weight, probably of the order 350.

Table III summarises spectroscopic data on certain of the simpler unsaturated compounds with conjugated ethenoid linkages, the data on the acids being due to Kuhn (private communication) and on the hydrocarbons to

Table III.

Acids		λ max. $m\mu$	$\log \epsilon$ max.
Crotonic	$\text{CH}_3\text{CH}=\text{CHCOOH}$	204	4.085
Sorbic	$\text{CH}_3(\text{CH}=\text{CH})_2\text{COOH}$	254	4.398
Octatrienecarboxylic	$\text{CH}_3(\text{CH}=\text{CH})_3\text{COOH}$	294	4.556
Decatetraenecarboxylic	$\text{CH}_3(\text{CH}=\text{CH})_4\text{COOH}$	329	4.690
Hydrocarbons			
$\alpha\alpha'$ -Diphenylbutadiene	$\text{C}_6\text{H}_5(\text{CH}=\text{CH})_2\text{C}_6\text{H}_5$	325	3.65
$\alpha\alpha'$ -Diphenylhexatriene	$\text{C}_6\text{H}_5(\text{CH}=\text{CH})_3\text{C}_6\text{H}_5$	371	3.8
		355	3.83
		333	3.7
$\alpha\alpha'$ -Diphenyloctatetraene	$\text{C}_6\text{H}_5(\text{CH}=\text{CH})_4\text{C}_6\text{H}_5$	396	3.87
		372	3.86
		357	3.80
$\alpha\alpha'$ -Diphenyldodecahexaene	$\text{C}_6\text{H}_5(\text{CH}=\text{CH})_5\text{C}_6\text{H}_5$	441	3.73
		418	3.73
		380	3.7
		317	3.2

Rădulescu and Bărbulescu [1931]. Two general tendencies are observable: (i) an increase in the number of conjugated double bonds causes a shift of the ultra-violet absorption maxima, or group of maxima, towards the visible; (ii) this shift is usually accompanied by an increase in absorption intensity. The groups of bands exhibited by the series of hydrocarbons due to the combined influence of ring systems and conjugated double bonds show a qualitative overlapping similar to that already mentioned in connection with the "fine structure" acids, indicating the possibility of a similar series relationship between the acids, the quantity present decreasing as the extent of conjugation increases. This would account for the general shape of the absorption curve of the total acids, which falls as the visible is approached, instead of rising as would be expected if equal quantities were present.

Table IV records the absorption maxima of a number of unsaturated substances connected with the whole question of fat-soluble vitamins. It will be observed that the bands in the visible associated with the highly unsaturated acids are reminiscent of those shown by carotenoids and polyene acids, whilst the ultra-violet bands resemble those shown by polycyclic, partially hydrogenated substances, the resemblance being closest for "dihydro" carotene (prepared by the action of aluminium amalgam on carotene) and the cyclised product derived from vitamin A and hydrochloric acid [Edisbury *et al.*, 1932]. The highly unsaturated acids probably contain appreciable quantities of substances with tricyclic skeletons, a view which receives some support when the absorption spectra of anthracene and phenanthrene derivatives are considered.

Table IV.

Total acids	—	460	445	422	401	—	377	360	348	332 <i>mp</i>
Vitamin A + HCl	—	—	—	420	—	392	—	369	350	333
" Dihydrocarotene "	—	461	426-45	—	—	392	—	369	350	332
Carotene	492	461	436	—	—	—	—	—	348	—
Bezssonoff's extract*	476	—	448	426	408	386	—	—	349	330
α -Croceetin	—	—	448	420-5	399	—	—	—	—	—
Dihydrocroceetin	—	—	—	—	—	—	382	361-5	344	—
Monk (angler-fish) pigment	480	—	450	422	—	—	—	—	—	—
Ergosterol	—	—	—	—	—	—	—	—	—	—
Dehydro-ergosterol	—	—	—	—	—	—	—	—	342	325-5
Ergosterol D	—	—	—	—	—	—	—	—	—	—

(continued)

Total acids	316-5	301-5	290	282	270	259	—	—	230-5 <i>mp</i>	
Vitamin A + HCl	313-20	—	292	280	272	261	254	—	—	
" Dihydrocarotene "	316	301	—	—	—	—	—	—	—	
Carotene	—	—	—	280	—	—	—	—	—	
Bezssonoff's extract*	—	300	—	283	—	—	—	—	—	
α -Croceetin	310	—	—	—	—	—	249-5	—	—	
Dihydrocroceetin	320-6	—	—	—	274-5	—	—	—	—	
Monk (angler-fish) pigment	—	—	—	—	—	—	—	—	—	
Ergosterol	—	—	293	281-5	270	260	250-3	242	—	
Dehydro-ergosterol	311	—	296	—	—	—	—	—	—	
Ergosterol D	—	—	—	—	—	—	252	243	235	

* A residue, kindly furnished by Prof. Bezssonoff, from carotene extracts.

The position now reached may be stated briefly.

1. The well-defined narrow absorption bands owe their origin to unsaturation in the absorbing molecules.
 2. The highly absorbing acids are not present in the original oils as the corresponding glycerides.
 3. The hypothesis that the absorbing acids are straight chain compounds with conjugated double bonds is not in harmony with the evidence.
 4. A change, unsaturated acids (low absorption) \rightarrow unsaturated acids (high and selective absorption) occurs during saponification.
 5. Cyclisation is the most plausible explanation of this change.
- Further work is proceeding on these lines.

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CXCVII. VARIATIONS IN VITAMIN A CONTENT OF FISH-LIVER OILS, WITH PARTICULAR REFERENCE TO SEASONAL FLUCTUATIONS IN THE POTENCY OF HALIBUT-LIVER OIL.

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(Received July 14th, 1933.)

THE object of the present work was initially to gather data concerning the vitamin A potency of the liver oils obtained from various species of fish. It was soon found that the vitamin A content of halibut-liver oil was remarkably high in comparison with oils from other species, and the scope of the investigation [*cf.* Lovern, 1932] was widened to embrace the elucidation of the factors capable of influencing the vitamin A content.

Collection of material. Livers were obtained mainly from commercial line-fishing vessels operating from Aberdeen and fishing in waters near Iceland, the Faroes and N.W. Scotland. The average duration of a trip was 2-3 weeks, and in each case the livers from all catches were mixed and stored at 0° in tins holding about 22 lb. The livers were received in varying states of preservation; some were very fresh and firm, whilst in other cases the contents of a tin had become a semi-liquid mass from which individual livers could not be separated. Each batch of livers was minced, mixed well, dried with anhydrous sodium sulphate and extracted with ether, and a dark sticky oil was finally obtained.

The halibut livers varied in weight from 4 oz. to 7 lb., but the great majority were between the limits of 12-20 oz., so that 1 lb. represents the approximate average weight of the halibut livers obtained by us in Aberdeen.

Towards the end of 1932 it became impossible to purchase halibut livers directly, as the production had been acquired in advance for the commercial extraction of the liver oil, but through the courtesy of Messrs Isaac Spencer and Co., Ltd., and Messrs Allen and Hanburys, Ltd., quantities of liver (10-14 lb. at a time) were purchased periodically. During the earlier period when supplies were obtained direct from the trawlers, arrangements were made for the skippers to note the position of the fishing ground, the nature of the stomach contents and the size of the halibut represented by the catch. As a rule, the livers in a batch were of assorted sizes, but occasionally fairly homogeneous catches of large or small halibut were obtained and the livers were classified as "mainly large" or "mainly small." The position of fishing grounds was also ascertained for the livers purchased from commercial houses during the later period. Through the courtesy of Messrs Parke, Davis and Co., we were also able to examine representative samples of commercial halibut-liver oil prepared from the livers of fish caught in Pacific waters and in the Davis Straits (*vide infra*). Much of

the data obtained is unsuitable for a detailed report, but the following main conclusions may be quoted.

(i) No very precise correlation between vitamin A potency and the position of the fishing ground can be established, although there are indications that higher maximum values are obtained in the liver oils from halibut caught in more northerly waters, probably reflecting the tendency of the larger fish to migrate northwards.

(ii) No significance can be attached to the stomach contents or the intensity of feeding [see Lovern and Sharp, 1933].

(iii) There is considerable evidence that the vitamin A potency of halibut-liver oils (in common with those of several other species) increases with increasing size of liver, and therefore age of fish.

(iv) Well marked seasonal variation was shown.

Vitamin A tests. Rough Carr-Price determinations were carried out at Aberdeen on the fresh oils, followed by spectrometric assay at Liverpool, using the Hilger-Nutting spectrophotometer for the antimony trichloride colour test, and Hilger E 3 spectrographs for the direct determination of vitamin A content on the basis of intensity of absorption in the ultra-violet shown by alcoholic solutions of liver oil.

Of all the constituents of fish-liver oils, vitamin A is by far the most variable in concentration. This is true not only in comparing oils from different species, but also as reflecting the differences between specimens of oil obtained from individuals of the same species. Our experience makes us unable to attach any precise significance to the idea of a fish-liver oil "typical" as regards vitamin A potency.

Variations in vitamin A content from species to species, arising possibly from different requirements and feeding habits, are perhaps only to be expected, but their magnitude is nevertheless surprising (see Table I). The percentages are calculated on the basis of the richest vitamin A distillates [*cf.* Carr and Jewell, 1933]:

$$E_{1\text{cm.}}^{1\%} \left. \begin{array}{l} 617m\mu, 5000 \\ 580m\mu, 2600 \\ 328m\mu, 1600 \end{array} \right\} \begin{array}{l} \text{antimony trichloride colour test.} \\ \text{direct ultra-violet absorption test.} \\ \text{78,000 Carr-Price units.} \end{array}$$

The samples of oil from some species represent only a relatively small number of livers, so that considerable variations from our figures may be possible in later tests. Nevertheless, experience up to the present points to the conclusion that the liver oils of haddock, whiting, skate of small or medium size, codling and immature or small fish generally are markedly inferior in vitamin A potency to average cod-liver oil; the oils from pollack, saithe, hake and ling (probably also torsk) are usually similar in potency to cod-liver oil and subject to roughly the same variations; salmon, turbot, sturgeon and halibut yield liver oils which are vastly richer in vitamin A than cod-liver oil. The richer the source the more widely does the potency appear to vary as between sample and sample.

According to the data of Bills [1927] the over-all range of antirachitic activity of liver oils is some 500:1, the majority of oils falling within a range of 30:1. Vitamin A has a wider range, at least 2500:1, and, so far as we can ascertain, no parallelism between vitamin A and vitamin D potencies can be substantiated. At present, we have no views to submit regarding the significance of a 2500:1 range in vitamin A content; we are more concerned to ascertain the facts relating to variations (within a given species) in batches of oil, each representing

Table I. *Vitamin A content of fish-liver oils.*

Liver oil	Estimated percentage of vitamin A in the samples examined
Catfish (<i>Anarrhichas lupus</i>)	0.06
Cod (<i>Gadus callarias</i>) (mean of 43 samples)	0.04
Codling (<i>G. callarias</i>) (smaller specimens)	0.004
Codling (<i>G. callarias</i>) (larger specimens)	0.008
Haddock (<i>G. aeglefinus</i>)	0.004
Pollack, lythe (<i>G. pollachius</i>)	0.04
Saithe, coalfish (<i>G. virens</i>)	0.09
Whiting (<i>G. merlangus</i>)	0.005
Conger eel (<i>Conger vulgaris</i>)	0.25
Dab (<i>Pleuronectes limanda</i>)	0.015
Lemon sole (<i>P. microcephalus</i>)	0.07
Plaice (<i>P. platessa</i>)	0.1
Hake (<i>Merluccius vulgaris</i>)	0.04
Halibut (<i>Hippoglossus vulgaris</i>) (highest recorded, 1932)	10.0
(<i>H. vulgaris</i>) (lowest recorded, 1932)	0.17
Ling (<i>Molva vulgaris</i>)	0.015
Monk (angler-fish) (<i>Lophius piscatorius</i>)	0.02
Newfoundland flat-fish (<i>Platysomichthys hippoglossoides</i>)	0.04
Pike (<i>Esox lucius</i>)	0.08
Rat-fish (<i>Chimera mirabilis</i>)	0.008
Salmon (<i>Salmo salar</i>)	0.3
"Blue" skate (<i>Raja batia</i>)	0.007
Thornback skate (<i>R. clavata</i>) (small specimens)	0.01
(<i>R. clavata</i>) (large specimens)	0.05
Sturgeon (<i>Acipenser attilus</i>)	0.8
Torsk, tusk (<i>Brosimius brosme</i>)	0.08
Turbot (<i>Psetta (Rhombus) maximus</i>)	0.3

too many livers to have any meaning as regards individual fishes but large enough, it is hoped, to be significant as regards the major causes of fluctuations and variations in potency.

Two distinct kinds of variation are discernible, progressive and seasonal, the seasonal being superimposed on the progressive variation; and without excluding the possibility of less obvious factors, the following variables appear to be important:

- (i) Size (or age) of fish.
- (ii) Sexual condition.
- (iii) Diet.

The progressive aspect of the vitamin A variability seems to depend on (i), whilst (ii) and (iii) appear largely to account for the seasonal changes.

Within a given species we have found that large livers yield as a general rule a more potent oil than small livers, and our qualitative observations are supported by the recent more quantitative work of MacPherson [1933], who has found a linear relationship between age of fish and the potency of cod- and American plaice- (*Hippoglossoides platessoides* Fabricius) liver oils. As the livers of the larger members of a species usually represent a greater proportion of the total body weight than is the case with smaller specimens, and as, further, the larger livers generally yield a higher percentage of oil, the total vitamin A reserve of the fish increases rapidly with size. This probably results from a purely mechanical storage of the excess of vitamin (ingested as such or synthesised *in vivo* from carotene) over the requirements of the fish. Be the explanation what it may, the age factor is of considerable importance. Thus in the cod, the ratio between vitamin A potencies for mature fish and codling is about 10:1,

corresponding to about 20 or 30:1 in the actual weight of vitamin A per unit weight of fish. Qualitatively confirmatory evidence is available for skate and halibut.

It has long been recognised that during the spawning period changes occur which may profoundly affect the vitamin content of the liver oil. The cod, for example, eats little or no food at such times, and the drain on fat-reserves incidental to fasting is increased by the utilisation of large quantities of liver oil in the development of the genital products [*cf.* Hjort, 1914]. A certain amount of vitamin A is transferred with this oil to the gonads [Zilva *et al.*, 1924], and the total vitamin reserves are to that extent depleted; but the residual liver oil is so reduced in quantity that the concentration of vitamin A in the oil is higher than when the fish is feeding normally. It appears, in fact, that at the spawning period both vitamin A and D potencies vary approximately inversely as the oil content of the liver [Drummond and Hilditch, 1930]. This utilisation of liver oil at spawning would of itself give rise to a peak in the potency curve immediately after spawning. Resumption of feeding results in gradual dilution of the liver oil as fat is again stored, the drop in potency being mitigated by replenishment of vitamin A reserves. The average percentage of oil in fish-livers varies from species to species (*e.g.* halibut 18–20 % (average); monk or angler fish, 40 %; certain gadoid fishes, up to 70 %) as also does the range over which the percentage of oil varies for the pre- and post-spawning periods, but the range rarely exceeds 2 or 3 to 1 for the spawning effect. The males of the various species are much less affected than the females.

Diet may next be considered as a contributory cause of fluctuations in potency. In a sense, diet is fundamental, since there is no evidence of direct synthesis of vitamin A by fishes as appears to be possible with vitamin D in some species [*cf.* Bills, 1927], and in any event the richer the diet, the greater are the vitamin reserves likely to be. Drummond *et al.* [1922], Jameson *et al.* [1922] and Hjort [1922] traced the fish-liver vitamin A through a series of intermediate stages to the diatoms (unicellular vegetable organisms in the plankton), which are able like land plants to produce carotene as a result of photosynthesis. There is as yet no evidence that diatoms or the minute planktonic animal organisms (copepods, larval forms of various marine creatures, *etc.*) which subsist on diatoms, contain vitamin A as such, or vitamin D. The published biological assays of the growth-promoting effects of planktonic organisms exhibit curiously good agreement in some cases, and very bad agreement in others, so that seasonal variations may perhaps be suspected here also. In any case, the plankton exhibits very well-marked seasonal fluctuations in abundance, an effect due primarily in the case of diatoms to seasonal changes in the intensity of sunlight and less obviously to fluctuations in silica, nitrogenous matter and carbon dioxide in the sea water. The curves in Fig. 1 show the mean annual changes in the abundance of planktonic flora and fauna generally in Port Erin Bay for the period 1907–1920, based on data given by Johnstone *et al.* [1924], and are fairly representative for the whole of the northern hemisphere, with the reservation that in 1932 the spring maximum of the diatoms may have been a few weeks earlier than usual (in Plymouth Sound this occurred in March). It would appear from the curves that the greater part (probably 80 %) of the total amount of carotene annually produced by the diatoms is synthesised in the spring or early summer, with a comparative scarcity, so far as initial synthesis is concerned, during the rest of the year. A time-lag of uncertain duration must exist before this carotene reappears in the form of vitamin A in the liver oils of large fishes, owing to the number of links in the

food-chain. The spring maximum must first of all affect the copepods and larvae living on diatoms; next, immature fish and small mature fish (*e.g.* herring, caplin), certain molluscs and other organisms which subsist on copepods; thence, either directly or through one or two further stages, the larger fish. The effect is, moreover, liable to be wholly or partly obscured by other factors such as the presence of large reserves of liver oil and changes in oil content due to spawning. Indeed, the last factor gives rise to the only seasonal fluctuation which can be discerned at all in the majority of species. The halibut, apparently, is an exception.

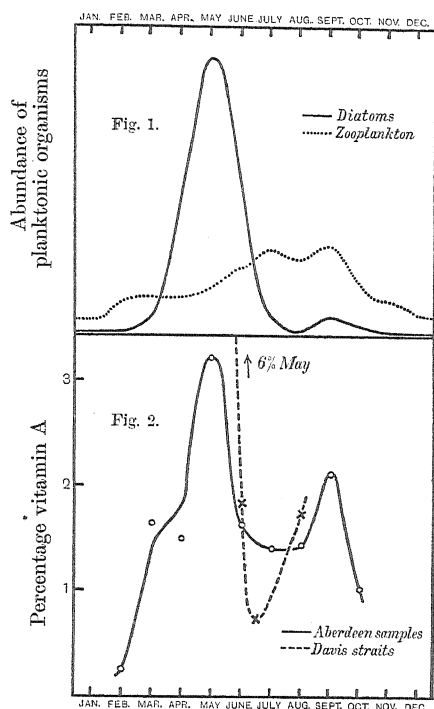


Fig. 1. Mean annual plankton variations, 1907-20, Port Erin Bay; adapted from Johnstone *et al.* [1924].

The diatom curve is roughly quantitative. The zooplankton curve, which is more diagrammatic, reflects the July and September maxima due to copepods, the main curve being modified by larvae (early spring and November) and protozoa (June-July).

Fig. 2. Seasonal variations in vitamin A content of halibut-liver oils (1932).

Insufficient samples were obtained during March to justify drawing the curve with a further subsidiary maximum in the early spring.

Tables II and III summarise data which have been obtained during 1932 on halibut-liver oils. Some comment must be made on the statistical significance of the results in the last columns of Tables II and III, from which the curves in Fig. 2 were drawn. The halibut livers from Aberdeen are representative samples of catches made during the greater part of the year, and not less than 1500 livers were worked up on a laboratory scale over the whole period. The material from the Davis Straits was obtained from some 100,000 livers (4500

Table II. *Halibut-liver oils (samples from Torry Research Station).*

Mean date of catch	Approx. total wt. of livers per batch in lbs.	Carr- Price B.U.	$E_{1\text{cm.}}^{1\%}$ 617m μ	$E_{1\text{cm.}}^{1\%}$ 580m μ	$E_{1\text{cm.}}^{1\%}$ 328m μ	Esti- mated % vita- min A	Aberdeen samples % vitamin A for month (weighted mean)
12. ii. 32	30	—	10.8	7.7	5.5	0.30	0.26
15. ii. 32	30	—	12.1	8.7	6.0	0.34	
22. ii. 32	30	60	7.5	6.0	4.25	0.23	
23. ii. 32	30	60	6.2	4.8	3.5	0.17	
7. iii. 32	30	—	70	47	30	1.9	1.65
10. iii. 32	30	1600	139	80	42	2.8	
16. iii. 32	30	71	10	6.5	5.0	0.25	
5. iv. 32	30	66	8.2	5.5	—	0.2	
18. iv. 32	30	1030	—	—	—	ca.2	1.5
18. iv. 32	30	1100	—	—	—	ca.2	
24. iv. 32	33	820	67	44	26	1.6	
27. iv. 32	16	540	42	29	19	1.1	
28. iv. 32	33	1900	132	89	50	3.1	
30. iv. 32	44	210	17.3	14.6	—	0.5	
3. v. 32	44	205	25	14.5	80	0.5	3.23
16. v. 32	30	2770	176	98	57.5	3.6	
17. v. 32	33	3030	241	194	80	5.0	
18. v. 32	22	7630	430	260	164	10.0	
20. v. 32	30	780	72.7	44.5	24	1.5	
21. v. 32	30	865	90	49	27.5	1.7	
4. vi. 32	30	285	18	14	8.5	0.53	1.63
10. vi. 32	30	890	113	75	39.3	2.5	
12. vi. 32	30	625	45	29.5	15.4	1.0	
14. vi. 32	30	1240	121	74	36.5	2.5	
1. vii. 32	30	290	20.8	12.4	6.8	0.42	1.41
3. vii. 32	30	1170	124	73	35	2.45	
16. vii. 32	30	1800	175	101	47.5	3.4	
18. vii. 32	77	775	85	52	23	1.7	
30. vii. 32	200	500	52	33.5	15	1.0	1.44
3. viii. 32	—	270	27	17	8.6	0.55	
3. viii. 32	—	1365	148	90	—	3.1	
3. viii. 32	33	480	58	35	19	1.2	
9. viii. 32	14	125	16.4	9.7	6.4	0.38	
9. viii. 32	14	1870	188	108	58	3.75	
10. viii. 32	—	1340	165	98	45	3.3	
10. viii. 32	—	385	34	22	11	0.75	
12. viii. 32	44	210	17.5	12.5	8.75	0.53	
14. viii. 32	—	3000?	134	81	42	2.8	
15. viii. 32	132	950	77	44.5	24.4	1.6	
19. viii. 32	14	240	20.2	14	8.7	0.5	
24. viii. 32	14	525	58	34	19.2	1.25	
30. viii. 32	110	355	37	20	12.3	0.8	
7. ix. 32	14	1080	83	50	25.5	1.7	2.12
9. ix. 32	30	1510	159	87	50	3.2	
9. ix. 32	14	685	78	44	24	1.6	
12. ix. 32	14	640	61.5	34	21.7	1.3	
13. ix. 32	14	1640	126	72	41.2	2.7	
22. ix. 32	14	610	54	34	16.6	1.1	
24. ix. 32	14?	165	13	8.5	5.5	0.33	
30. ix. 32	44	1750	166	94.5	48.7	3.2	
30. ix. 32	166	1000	98	55	30	2.0	
30. ix. 32	—	1740?	102	60	32	2.2	
30. ix. 32	—	975	71	44.5	24	1.55	
30. ix. 32	—	895	59	34.6	19	1.25	
30. ix. 32	—	800	52.4	30.4	17.1	1.1	1.02
30. ix. 32	—	1470	86	52	29.5	1.8	
7. x. 32	14	385	34	24	15.3	0.95	
13. x. 32	14	420	40	24	14.5	0.90	
21. x. 32	14	160	13.9	8.8	5.4	0.32	1.9
26. x. 32	—	1530	90	50	31	1.9	

Table III. *Halibut-liver oils from the Davis Straits.*

Fish caught (1932)	Carr-Price B.U.	$E_{1\text{ cm.}}^{1\%}$ 617 $m\mu$	Vita-min A % 6.0	$E_{1\text{ cm.}}^{1\%}$ 580 $m\mu$	Vita-min A % 6.54	$E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$	Vita-min A % 5.62	Mean % vita-min A 6
May	4700	300	6.0	170	6.54	90	5.62	6
June	1400	90	1.80	49	1.88	28.7	1.80	1.83
June-July	630	37	0.74	19	0.73	12	0.75	0.74
August	1150	85.4	1.71	47	1.81	27.5	1.72	1.74

tons of fish) and the livers worked up on a technical scale. The results of the small scale and large scale treatments are consistent. The monthly average vitamin A potencies of the samples obtained from Aberdeen exhibit a very sharp maximum in May and a subsidiary maximum in September, and the curve for the Davis Straits samples is qualitatively similar over the period for which the data are available.

DISCUSSION.

The interpretation of Fig. 2 is a matter of some difficulty. The observed fluctuations in monthly average potency cannot be attributed entirely to variations in oil content due to spawning. In the first place, the range of potency covered by the Davis Straits samples is about 8:1, whereas all the oils examined have represented between 12 and 35 % of the liver weight (*i.e.* a range of 3:1), the great majority lying between 15 and 25 %. Secondly, the seasonal fluctuation in potency is just as marked and shows the same tendency to the same two maxima whether spawning occurs in February-May (N.W. Scotland) or June-August (northern waters). For this reason all the data for the Aberdeen oils have been combined in one curve, whether the fish were caught off Iceland, the Faroes, or Scotland, and the "statistical" halibut represented by this curve may be regarded as spawning over the whole period from February to August.

Unless there is some more obscure process operating on or within the halibut, the only remaining likely cause of seasonal fluctuations in potency is the variation in available carotene, and it seems significant that the only measure we have of this factor, namely the abundance of diatoms, should show, qualitatively at all events, such a strikingly similar curve (Fig. 1). We hesitate to stress the parallelism unduly without further confirmatory evidence, and a completely convincing correlation is naturally very difficult to achieve since the halibut does not subsist directly on diatoms. Nevertheless, it seems to us highly probable that when carotene (in the plankton) is abundant, vitamin A reserves are being replenished by all fish, and that when these supplies are small the reserves are being drawn upon. The effect on liver oil potency will not be very marked in most fish, but the halibut appears to reflect this ebb and flow in so marked a way as to obscure even the spawning factor. The fact that the vegetable plankton is richer in colder waters and that the halibut-liver oils from the Davis Straits reached in May an average potency nearly twice as high as the Aberdeen samples, is not inconsistent with this view, though the larger size of fish may also account for the phenomenon. If the latter view is correct, it would appear that the larger the fish, the greater the fluctuation, as is shown by the low minimum reached by the Davis Straits curve.

The curves further suggest an enormous consumption of reserve vitamin A by the halibut during the summer and late autumn, and the possibility cannot at present be excluded, as an alternative explanation, that the potency fluctuation may arise from a rhythmic transference of vitamin A between the liver

and some other part of the body, influenced by some unknown factor which varies seasonally in the same way as the diatoms. All this, of course, is pure speculation, but whatever their origin the fluctuations themselves seem real enough. It remains to be seen whether the halibut-liver oil curves published here are reproducible from year to year, subject, of course, to minor variations in time and intensity. We have, however, noticed the same general tendency for two successive years, and there is no reason to believe that they were exceptional.

The over-all range in variation of vitamin A in halibut-liver oils is seen from Table II to be certainly not less than 60:1 (10 to 0.17 %); more probably, since the samples each consisted of mixed oils from a number of livers, nearer 100:1, a much larger range than has been observed in any other species. Seasonal fluctuations account for only a small proportion of this range, and variations in the age of the fish caught (which, we are informed, would be from about 12 to 40 years) may here again be the dominant factor, as it appears to be in the case of cod [MacPherson, 1932; 1933].

We are, in short, led to infer that the oil obtained from any given halibut liver increases in mean annual potency as the fish grows older, and in addition fluctuates from season to season over a gradually increasing range. Further, the extraordinarily high proportion of vitamin A in some of the samples, together with these wide fluctuations in concentration, suggests that, at least in the halibut metabolism, vitamin A may play an additional rôle to that usually associated with the term "vitamin."

SUMMARY.

1. The vitamin A content of fish-liver oils covers a range of at least 2500 to 1. No parallelism can be traced between the vitamin A and vitamin D potencies.
2. In certain species, the vitamin A content of the liver oil has been found to increase with the age and/or size of the fish, the total vitamin A reserve increasing more rapidly than the oil potency.
3. Attention is drawn to the fact that the greater part of the carotene annually produced by the diatoms, and thus available for conversion into vitamin A by marine animal life, is initially synthesised during a comparatively short period in the spring or early summer.
4. Halibut-liver oil is by far the richest known natural source of vitamin A available in quantity, but it has been found to vary in potency over a wider range than any other source. Oils containing from 0.17 to 10 % of vitamin A have been examined and these apparently do not represent extreme limits. No correlation has emerged between the immediate diet of the halibut and the oil potency [Lovern and Sharp, 1933].
5. Halibut-liver oils have been found to exhibit well-marked seasonal fluctuations in vitamin A concentration which cannot be attributed to changes in the oil content of the liver occasioned by spawning. The explanations offered are necessarily tentative, but the fluctuations themselves are quite definite.
6. The best oils from the standpoint of vitamin A content are most likely to be obtained from large halibut caught in northern waters in the late spring or early summer, and in the autumn. Very rich oils at other times of the year are exceptional. (*Mutatis mutandis* this may also apply to the southern hemisphere.)

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CXCVIII. THE DIET OF THE HALIBUT AND INTENSITY OF FEEDING, IN RELATION TO THE VITAMIN A POTENCY OF THE LIVER OIL.

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(Received July 14th, 1933.)

In an endeavour to elucidate the factors responsible for the observed wide variations in the vitamin A potency of halibut-liver oil [Lovern *et al.*, 1933], examinations were made of the stomach contents of a number of fish. The method adopted was to furnish the skippers of the vessels supplying the livers with a typewritten form, on which, amongst other information, they wrote down the nature of the stomach contents (as far as ascertainable) of all their catch of halibut. Any material which they could not readily identify was placed in bottles of formalin, supplied for the purpose, and brought back for identification at the Torry Research Station.

It was realised from the outset that food found in the stomachs only gave evidence of the nature of the last meal, and was no clue to the type of food on which the fish had been feeding for some time. However, it was hoped that some light might be thrown on the source of the unusually large supplies of vitamin A present in the liver of the halibut—even in the poorest specimens. The results, however, were entirely negative. In many cases the stomachs were empty, but the actual food found was of so varied a nature as to suggest that the halibut ate anything they could get. The commonest species found in the stomachs were torsk, dogfish, catfish, small dabs, and crustaceans, with many other specimens, such as megrims, ling, *Sebastes norvegicus*, etc. In some cases remains of herring were found, but it could not be decided whether these had come from the bait or not.

It was evident from the vitamin A potencies of the liver oils (as measured by the antimony trichloride reaction) that no relationship could be adduced between high potency and any particular diet. None of the species found in the stomachs is an unusually rich source of vitamin A, although the one sample of torsk-liver oil which was examined was superior to most cod-liver oils. It may be worth noting that torsk was found in the stomachs more frequently than any other species.

As the diet seemed to be of so general a nature, with no outstandingly rich supply of vitamin A, it was decided to try to get some measure of the intensity of feeding. For this purpose, the glycogen content of the livers was chosen as a criterion. Admittedly it has obvious drawbacks. It is not known, for instance, to what extent intensive feeding, on a diet mainly of proteins and some fat, would affect the liver glycogen content, nor is it known to what extent, or how quickly, struggling of the fish on the line would deplete the liver reserves of

glycogen. In any event, wide variations in glycogen content were experienced, so large, in fact, that it seems the most feasible explanation to ascribe them to dietary causes. Even so, it is extremely doubtful if the values have reference to anything more remote than the last meal of the fish, but no other means of investigating intensity presented itself.

From fish brought aboard a great-line vessel fishing the Faroes grounds the livers were immediately cut out and weighed, a sample of each being preserved in 60 % KOH for estimation of glycogen on return of the vessel to port. The remainder of the livers, used for oil and vitamin A analyses, was stored in ice in two batches; A, all livers under $\frac{3}{4}$ lb. in weight, B, all livers over $1\frac{1}{2}$ lbs. in weight. The data obtained from the first two experiments are given in Table I. Vitamin A potencies are expressed as blue units per 0.2 cc. of 20 % solution.

Table I.

Trip No.	Weight of whole livers (lbs.)	No. of livers in lot	Variation in glycogen (g. per 100 g. liver)	Average glycogen (g. per 100 g. liver)	Oil (%)	Vitamin (blue units)
1	< $\frac{3}{4}$	17	Trace-7.47	1.57	16	269
	> $1\frac{1}{2}$	10	Trace-4.78	1.71	18	1366
2	< $\frac{3}{4}$	21	0.04-0.90	0.45	18	386
	> $1\frac{1}{2}$	8	0.05-0.79	0.33	20	1337

So great were the individual differences in glycogen values that in two later experiments a somewhat different procedure was followed. In place of storing the remainder of the livers in two large batches, each liver was stored separately until the glycogen values were obtained. The livers were then put into lots according to glycogen concentration. The results are given in Table II.

Table II.

Trip No.	Weight of whole livers (lbs.)	No. of livers in lot	Variation in glycogen (g. per 100 g. liver)	Average glycogen (g. per 100 g. liver)	Oil (%)	Vitamin (blue units)	Mean
3	< $\frac{3}{4}$	8	0.01-0.18	0.09	12.5	975	1080
		6	0.22-0.98	0.49	12.0	1469	
		6	1.58-3.27	2.2	18.1	797	
	> $1\frac{1}{2}$	5	0.13-0.62	0.38	22	1742	1319
		3	1.48-1.82	1.67	22	897	
4	< $\frac{3}{4}$	8	Trace-0.56	0.27	20	283	407
		4	0.72-1.67	1.12	18	699	
		4	2.92-6.50	4.28	18	238	
	> $1\frac{1}{2}$	4	Trace-0.27	0.13	25	663	583
		3	0.79-1.02	0.94	18	550	
		3	3.92-6.35	5.04	20	537	

The tables show clearly that there is no relation between the vitamin potency of the oil and the glycogen content of the liver.

For each experiment the mean value for vitamin A potency is lower for the small livers than for the large ones, this being further evidence in favour of the conclusions already arrived at [Lovern *et al.*, 1933] regarding age of fish as a factor influencing liver oil vitamin A potency.

SUMMARY.

(1) The diet of the halibut is of a general nature, with no outstandingly rich source of vitamin A to account for the high potency of halibut-liver oil.

(2) Taking the glycogen content of the liver as a criterion of intensity of feeding no correlation could be established between intensity of feeding and the vitamin A potency of the oil.

(3) Further evidence was obtained that in general the livers of older fish afford a more potent oil than those of younger fish.

REFERENCE.

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CXCIX. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXIV. A NOTE ON THE MECHANISM OF THE PRO- DUCTION OF PHENOLIC ACIDS FROM GLUCOSE BY *PENICILLIUM BREVI-COMPACTUM* DIERCKX.

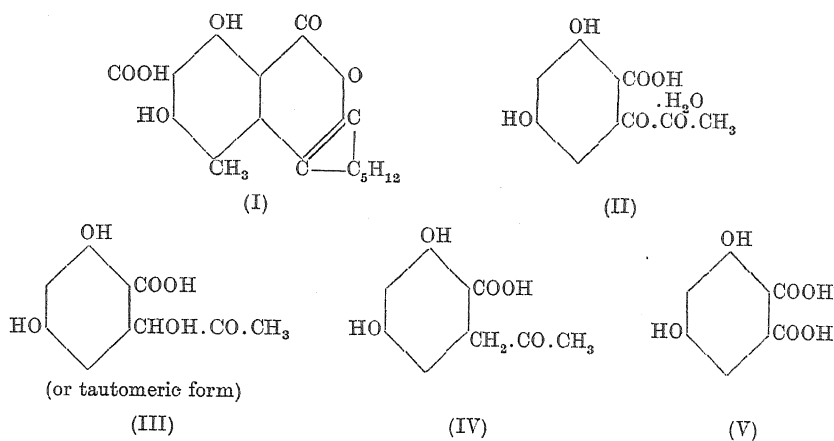
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(Received August 1st, 1933.)

It was shown in Part XXIV of this series [Clutterbuck *et al.*, 1932] that when most species and strains in the *P. brevi-compactum* Dierckx series are grown on synthetic media containing glucose, or glucose and tartaric acid, as sole source or sources of carbon, five phenolic acids are present in the metabolism solution when all the glucose originally present has been utilised by the mould. The empirical formulae of these five acids are, in descending order of complexity, mycophenolic acid, $C_{17}H_{20}O_6$ (I), $C_{10}H_{10}O_7$ (II), $C_{10}H_{10}O_6$ (III), $C_{10}H_{10}O_5$ (IV), and 3:5-dihydroxyphthalic acid, $C_8H_6O_6$ (V). Their respective constitutional formulae have been either completely or almost completely elucidated [see Clutterbuck and Raistrick, 1933; Oxford and Raistrick, 1932; 1933].

Monomethyl ether of



We may note here that all five acids are derivatives of resorcinol, and hence it seemed unlikely that all would prove to be stable end-products formed from glucose by five separate series of reactions, a more likely hypothesis being that some of these acids are intermediates in the formation of others. The present work was undertaken to discover the exact order of appearance of the phenolic metabolic products and the nature of the stable end-products of metabolism.

EXPERIMENTAL.

The strain of *P. brevi-compactum* Dierckx chosen (Catalogue Number M 3 (1)) was isolated by the late J. H. V. Charles in 1931 from mouldy Italian maize, and in that year gave relatively good yields of all five metabolic products when grown on Raulin-Thom medium [see Clutterbuck *et al.*, 1932, p. 1451]. 105 one-litre flasks each containing 350 cc. of Raulin-Thom medium of the following composition: glucose, 75 g.; tartaric acid, 4 g.; ammonium tartrate, 4 g. di-ammonium hydrogen phosphate, 0.6 g.; K_2CO_3 , 0.6 g.; $MgCO_3$, 0.4 g.; $(NH_4)_2SO_4$, 0.25 g.; $ZnSO_4 \cdot 7H_2O$, 0.07 g.; $FeSO_4 \cdot 7H_2O$, 0.07 g.; water to 1500 cc., were sterilised and to each were added 3 cc. of a well-shaken suspension of spores in sterile distilled water (total volume, 350 cc.) prepared from 4 beer wort agar slopes of the above organism which had been incubated for 36 days. The flasks, after inoculation, were incubated at 24°, and rapid growth took place, a complete felt of mycelium being formed in every flask after 3 or 4 days' incubation. 26 flasks were removed after 8 days when the addition of ferric chloride to a test portion of the metabolism solution first showed definitely that phenols were present. The mycelium and the filtered metabolism solution were dealt with separately, the following tests and estimations being carried out on a small average sample of the latter: coloration with ferric chloride; approximate p_H ; residual glucose by polarimeter; bromine absorption value in mg. per cc. (by Koppeschaar's method). The metabolism solution was then evaporated to 400 cc. *in vacuo* below 50° and worked up exactly as described by Clutterbuck *et al.*, 1932], except that the ether extraction at p_H 5.3 was omitted. The mycelium was dried, powdered and extracted in an all-glass Soxhlet apparatus first with boiling light petroleum (B.P. 50–60°) for 2 days, and then with ether for 2 days. It was found that when the mycelium contained mycophenolic acid, part of this acid was removed by light petroleum (in which it is very sparingly soluble) and crystallised out from the extract, the remainder being subsequently extracted by ether. The ethereal extract was evaporated, the residue was dissolved in a little alcohol and the mycophenolic acid isolated *via* the insoluble dipotassium salt.

The remaining flasks were taken off at the following intervals: 25 after 11 days; 20 after 15 days; 20 after 22 days (when all the glucose had been metabolised) and the remaining 14 after

Table I.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Period of incubation (days)	Glucose metabolised (g.)	Tests on metabolism solution					
		p_H	Bromine absorption value (mg. per cc.)	Coloration with ferric chloride	Tartaric acid recovered (g.)	Wt. of dried mycelium (g.)	Wt. of $C_{17}H_{20}O_6$ (I) (mycophenolic acid) (g.)
8	202 (54 %)	4	1.82	Not very intense brown	3.0	66	1.67 (63 % in mycelium)
11	269 (72.5 %)	4	2.80	More intense brown	0.8	83	2.28 (61 % in mycelium)
15	333 (90 %)	Between 4 and 5	3.86	Brownish-purple	0	87	2.67 (62 % in mycelium)
22	371 (100 %)	Between 4 and 5	4.64	Deep blue-crimson	0	78	2.85 (60 % in mycelium)
56	371 (100 %)	About 8	5.07	Deep blue-crimson	0	61	3.49 (all in metabolism solution; none in mycelium)

Notes: (a) Recrystallised from water, M.P. 120–132° (efferv.). Converted into 2:4-dinitrophenyl-
(b) The p_H 7 fraction weighed 3.3 g. of which 1.26 g. was $C_{17}H_{20}O_6$ (I). The rest (2.04 g.)

56 days. The last group of flasks was therefore incubated for 34 days after all the glucose had disappeared, the mycelial felts being partly waterlogged at the expiry of this period, and the metabolic products found in this final metabolism solution were, therefore, stable or relatively stable end-products of metabolism.

The results are summarised in Table I concerning which the following observations may be made.

(a) The data for each period of incubation are calculated on the basis of 20 flasks containing initially 371 g. glucose, 19 g. tartaric acid and 19 g. ammonium tartrate, the initial p_H of the medium being about 4.

(b) By tartaric acid recovered (column 6) is meant the weight of tartaric acid corresponding to the ether-insoluble part of the material precipitated during the evaporation of the metabolism solution to small bulk. This material gave strong reactions for potassium and for tartrates, and its very sparing solubility in water indicated that it consisted largely of potassium hydrogen tartrate.

(c) The weights of $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$ recorded in columns 9, 10 and 12 were isolated from the final fraction extracted by ether at a reaction acid to Congo red, the total weight of this fraction being given in column 13. The much smaller fraction extracted at p_H 7 was worked up for mycophenolic acid only but undoubtedly contained small amounts of the C_{10} compounds. This unavoidable loss accounts in part for the discrepancy between the weights recorded in columns 14 and 15.

The following points arise from a consideration of the results in Table I.

(a) Utilisation of glucose and utilisation of tartaric acid by *P. brevicompactum* take place concurrently. Although the presence of tartaric acid is necessary for good colour reactions in flasks it is not metabolised preferentially.

Table I (cont.).

(9)	(10)	(11)	(12)	(13)	(14)	(15)
Wt. of $C_{10}H_{10}O_6$ (III) (g.)	Wt. of $C_{10}H_{10}O_7$ (II) (g.)	Wt. of $C_{10}H_{10}O_5$ (IV)	Wt. of $C_8H_6O_6$ (V) (g.)	Wt. of "acid to Congo" fraction (g.)	Total wt. of crystal- line phenols isolated (g.)	Total wt. of crude phenols from which (14) was isolated (g.)
2.41	Not detected with certainty but probably present	Trace detected with certainty, M.P. 146- 150° efferv. remelts at 200-220°	Trace	3.56	4.08	6.0
3.69	0.13 (a)	Not looked for	0.03	5.79	6.13	9.32
4.05	1.3	Detected with cer- tainty	Not worked up	7.97	8.02	12.42
3.65	3.0	None	0.11	10.55	9.61	14.31
None	5.14	None	0.30	10.20	8.93 (b)	15.73 (b)

hydrazone, M.P. 210-212° decomp. after crystallisation from ethyl acetate-light petroleum. was necessarily lost in the estimation of $C_{17}H_{20}O_6$ (I).

(b) For some reason at present unknown the ferric chloride coloration given by the metabolism solution in the early stages of growth is of no service in predicting which phenolic substances are present.

(c) As long as the p_H of the metabolism solution is well on the acid side of neutrality, the greater part of the mycophenolic acid remains in the mycelium. This is due to the fact that mycophenolic acid is almost insoluble in cold water but is readily soluble in dilute aqueous alkali. The other phenolic metabolic products are, on the other hand, very much more soluble in cold water.

(d) The stable end-products of metabolism are mycophenolic acid (I), and the acids $C_{10}H_{10}O_7$ (hydrated form of 3:5-dihydroxy-2-carboxybenzoylmethylketone II) and $C_8H_6O_6$ (3:5-dihydroxyphthalic acid V), but the final yield of the last named is small compared with the yields of the other two acids. The acid $C_{10}H_{10}O_6$ (3:5-dihydroxy-2-carboxyphenylacetylcarbinol III) is definitely an intermediate product of metabolism since its maximum yield (relatively very considerable) is reached on about the fifteenth day, after which, during the next 40 days, it is completely metabolised. The acid $C_{10}H_{10}O_5$ (3:5-dihydroxy-2-carboxybenzylmethylketone (IV)) is never at any time present in other than trifling amount and appears not to be a stable end-product of metabolism.

DISCUSSION AND SUMMARY.

The results given in Table I definitely prove four series of facts, (a), (b), (c) and (d), and give fairly clear indication of another series (e).

(a) The yield of mycophenolic acid (I) increases continuously during the whole course of metabolism. A surprisingly large proportion, however (almost 50 %), of the total amount of mycophenolic acid (I) produced arises in the very early stages of metabolism (8 days after inoculation) and before growth of the organism is complete as is indicated by the weight of mycelium at this stage. Only a small proportion of the total yield of mycophenolic acid (less than 20 %) arises during the 34 days' incubation following the complete disappearance of glucose from the metabolism solution.

(b) The rate of production of the acid $C_{10}H_{10}O_7$ (II) is very different from that of mycophenolic acid. In the early stages of metabolism the presence of the acid $C_{10}H_{10}O_7$ could not be detected with certainty, and even after 11 days' incubation when 72.5 % of the glucose had been metabolised and growth of the mould had almost reached a maximum, only about 2.5 % of the final yield of the acid $C_{10}H_{10}O_7$ had been produced. On the other hand, about 40 % of the total amount of the acid $C_{10}H_{10}O_7$ produced arises towards the end of the incubation period, *i.e.* during the 34 days' incubation following the complete disappearance of glucose from the medium.

(c) The yield of the acid $C_{10}H_{10}O_6$ (III) increases rapidly to a maximum which is reached after an incubation period of 15 days, by which time 90 % of the glucose has disappeared, and then decreases in amount until this acid has disappeared completely from the medium after 56 days' incubation. Like mycophenolic acid (I) and unlike the acid $C_{10}H_{10}O_7$ (II) this acid is produced in largest amount (about 60 %) in the very early stages of metabolism, *i.e.* after 8 days' incubation.

(d) 3:5-Dihydroxyphthalic acid (V) is never present in large amounts but increases steadily during the whole course of metabolism, and at least 60 % of the total yield is produced after all the glucose has disappeared from the medium.

(e) The acid $C_{10}H_{10}O_5$ (IV) is present in very small amounts in the early stages of metabolism, but is absent in the later stages, none being detected after 22 days' incubation.

It must be admitted that the above results were entirely unexpected and are not easy of explanation, though it is hoped to obtain further light on the subject by a continuation of the work on somewhat different lines.

In view of the facts that mycophenolic acid (I), the acids $C_{10}H_{10}O_7$ (II), $C_{10}H_{10}O_6$ (III), $C_{10}H_{10}O_5$ (IV) and 3:5-dihydroxyphthalic acid (V) are all resorcinol derivatives, and that all have carbon side-chains in the 1 and 2 positions, it was natural to assume that one series of reactions or one set of precursors would be common to them all. The results presented do not offer conclusive evidence that this is the case.

We may, however, conclude with reasonable certainty that 3:5-dihydroxyphthalic acid (V) is an oxidation product of one or more of the other metabolic products.

Further, the fact that the acid $C_{10}H_{10}O_6$ (III) is produced in the early stages of growth and disappears completely in the later stages, while the acid $C_{10}H_{10}O_7$ (II) increases in amount throughout the whole course of metabolism and is produced in largest proportions during the later stages, renders it reasonably certain that the acid $C_{10}H_{10}O_7$ (II) arises by direct oxidation of previously formed $C_{10}H_{10}O_6$ (III). This change is indeed one which, as has already been shown [Oxford and Raistrick, 1933], can be readily brought about by purely chemical means. It is surprising to find that there should be a progressive accumulation of the acid $C_{10}H_{10}O_7$ (II), which is an α -diketone and which, certainly on purely chemical grounds, and probably on general biochemical grounds, might be expected to be readily oxidised by an organism whose available supplies of energy in the form of glucose have been exhausted.

The origin of the acid $C_{10}H_{10}O_5$ (IV) is not clear since the small yield and the difficulties of isolation render its estimation very difficult. It seems unlikely that the side-chain $—CH_2.CO.CH_3$ in this acid would be oxidised to $—CHOH.CO.CH_3$ in $C_{10}H_{10}O_6$ (III) more rapidly than the latter side-chain is oxidised to $—CO.CO.CH_3$ in $C_{10}H_{10}O_7$ (II), as must be the case if $C_{10}H_{10}O_5$ (IV) is the precursor of the other C_{10} acids. However, the ease with which it can be prepared *in vitro* from the acid $C_{10}H_{10}O_6$ (III) [Oxford and Raistrick, 1933] and the fact that it is present in the metabolism solution when the yield of the acid $C_{10}H_{10}O_6$ (III) is at a maximum, whereas it is absent when the acid $C_{10}H_{10}O_6$ (III) has disappeared and the yield of the acid $C_{10}H_{10}O_7$ (II) is at a maximum, indicate that it may be formed by the mould by reduction of the acid $C_{10}H_{10}O_6$ (III) and not by reduction of the acid $C_{10}H_{10}O_7$ (II).

It is tempting to postulate that the acid $C_{10}H_{10}O_6$ (III) is also the precursor of mycophenolic acid (I). The results presented do not exclude this possibility, but a consideration of the constitutional formulae assigned to these two acids shows that if this hypothesis is to be entertained certain assumptions must be made for which there are, so far as we are aware, no existing analogies in microbiological chemistry. Among other chemical changes necessary to convert $C_{10}H_{10}O_6$ (III) into mycophenolic acid (I) methyl and carboxyl side-chains must be introduced into the benzene nucleus. If, however, the possibility were admitted of introducing a $—CHO$ group into the benzene nucleus by a microbiological process, then the introduction of two $—CHO$ groups in the 4 and 6 positions into the molecule of the acid $C_{10}H_{10}O_6$ (3:5-dihydroxy-2-carboxyphenylacetylcarbinol, III) followed by the reduction of the $—CHO$ group in the 6-position to CH_3 , and the oxidation of the $—CHO$ group in the 4-position to $—COOH$, immediately leads to the substituted aromatic nucleus shown to be present in mycophenolic acid (I).

The possibility that mycophenolic acid (I) is the precursor of the three

C_{10} -acids, which might be supposed to be formed from it by oxidation, demethylation and decarboxylation, is rendered improbable by the fact that even after all the glucose has been metabolised the yields of both mycophenolic acid (I) and the acid $C_{10}H_{10}O_7$ (II) continue to increase. It must be remembered, however, that after the glucose has completely disappeared the mycelium decreases appreciably in weight, and hence the possibility cannot be ruled out entirely that the substances produced from the mycelium during this period might give rise to further amounts of mycophenolic acid (I) and/or the acid $C_{10}H_{10}O_7$ (II). Further, in view of the relatively large final yield of mycophenolic acid, this possibility would involve the assumption that, after all the glucose has been utilised, the mould loses to a very large extent its former power of oxidising mycophenolic acid.

Finally it must be pointed out that we have failed up to the present to find any indication as to what are the steps involved during the initial formation from glucose of the resorcinol nucleus common to all the five phenolic metabolic products of *P. brevi-compactum*, since investigation of the metabolism solution in the earliest stages of growth failed to reveal any products of metabolism other than the five phenolic metabolic products which we have described previously.

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CC. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

XI. THE MEASUREMENT OF RESPIRATORY QUOTIENT, RESPIRATION AND GLYCOLYSIS WITH THE AID OF THE CONSTANT-VOLUME DIFFERENTIAL MANOMETER.

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(Received August 22nd, 1933.)

THE methods previously described in this series make possible the accurate measurement of the true respiratory quotient and respiration of tissues suspended in a phosphate medium [Dickens and Šimer, 1930], or in bicarbonate media (including serum) in equilibrium with CO_2 at physiological tension [Dickens and Šimer, 1931]. Additional points of technique were described recently [Dickens and Šimer, 1933]. These methods may be applied to all types of tissue; but when the bicarbonate method is applied to tissues in which the aerobic acid production is high compared with the respiration, the glycolysis may not persist throughout the experiment at its initial level. For with such tissues, although it is adequate with others, the amount of bicarbonate solution which can be used may be unable to prevent an undue fall of p_{H} and increase of lactate concentration in the medium. With the simple manometer used, and also with the Barcroft manometer as applied to the measurement of respiratory quotient by Dixon and Keilin [1933], the permissible bicarbonate reserve is limited by the length of the manometer limb and the density of the manometer fluid. Increase of the former is inconvenient; increase in the latter results in loss of sensitivity. The difficulty has been overcome by the application of the constant-volume differential manometer [Dickens and Greville, 1933] to the respiratory measurements. It is now possible to use a sensitive manometer fluid (Brodie fluid of sp. gr. approximately 1), and to have a volume of bicarbonate solution such that, even with highly glycolysing tissues, the glycolysis remains nearly constant throughout. The conditions are strictly comparable with those of the Warburg [1924] two-vessel method; but whereas Warburg's method gives only the O_2 consumed and the total CO_2 produced by respiration and glycolysis, the method now described gives the true respiratory quotient and the true aerobic glycolysis, respiratory CO_2 and O_2 -uptake.

METHOD.

Principle. The constant-volume differential manometer has both vessels rigidly attached to manometer limbs which may be moved independently up and down. By this means the meniscus in each limb is kept at a fixed point in the

scale, and the contents of the vessel at constant volume; the difference in pressure between the vessels is given in terms of height of manometer fluid by the vertical distance between the menisci in the two limbs. Each limb has a tap which enables the vessels, the limbs and the outside air to be connected in pairs or all together. When this manometer is used for the measurement of R.Q., equal weights of tissue are put into equal volumes of medium in the two vessels, which are of equal size. Excess of acid is tipped into the medium in one vessel at the beginning of the experiment, in the other at the end. The pressure difference between the two vessels immediately before addition of acid to the medium in the first vessel is zero; after acid addition in the second vessel it is equal to the pressure increase due to the respiratory CO_2 less the pressure decrease due to the absorbed O_2 . The total CO_2 in both vessels is now absorbed, when the pressure difference between them represents the O_2 absorbed during the experiment. This subtracted from the previous pressure difference gives the respiratory CO_2 , so that the R.Q. can at once be calculated. Hence, the measurement of respiration and R.Q. necessitates only two readings; that of aerobic glycolysis, which is deduced from the pressure change in the second vessel during the experiment, demands a third reading in conjunction with a thermobarometer. The latter is unnecessary if only R.Q. and respiration are to be measured.

Apparatus. The constant-volume differential manometer, its use including filling with gas and its calibration have already been fully described [Dickens and Greville, 1933]. For the measurement of R.Q. the only modification lies in the use of a special vessel of new design¹ (Fig. 1). This is of conical shape and of

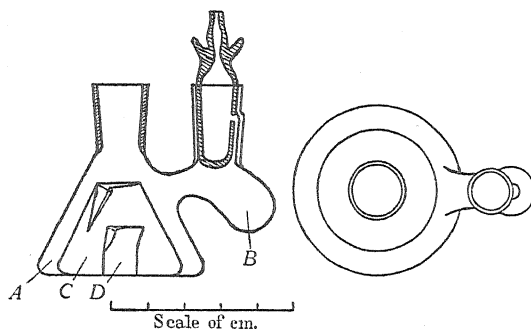


Fig. 1.

20–25 cc. volume. The diameter of the base should be about equal to the height: this gives stability to the vessel and large surface to the solutions. The vessel contains a separate inner conical part *C*, in the centre of the base of which there is a small cylindrical vessel *D* with a lip, like a small jug. *D* and *C* have each a groove at a suitable point in their rim to facilitate the insertion of pipettes and tissue. *B* is a bulb communicating with the outer part *A*; it has a bored stopper which can be closed by turning [Warburg and Kubowitz, 1929] and which is provided with hooks from which rubber bands may be stretched to hooks on the vessel. On inclining the vessel to the left (Fig. 1), liquid may be tipped from *B* into *A*; on inclining to the right, liquid first runs from *A* into *E*, and finally the contents of *D* drain almost completely into *C*.

¹ Made for us by Messrs C. Dixon and Co., 27, Devonshire Street, London, W.C. 1.

Having only one stopper, the vessel is more convenient and less likely to leak than that described for the original bicarbonate method [Dickens and Šimer, 1931]; it is also more stable; hence its use in that method is now recommended.

Procedure. The solutions are first measured into the various parts of the vessels as follows:

- A. Bicarbonate-containing medium (3.0 cc. are usually taken).
- B. 2.5 *N* hydrochloric acid, 0.25 cc.
- C. 43 % NaI, 2H₂O, 2 cc. (acidified immediately before experiment with 0.1 *N* sulphuric acid, methyl orange as indicator).
- D. 30 % NaMnO₄, 3H₂O containing *N*/400 H₂SO₄, 0.5 cc.

The medium must be accurately measured with a grease-free pipette with two marks and a fine tube; such a pipette delivered 3 cc. with an extreme error of ± 4 mg. in twelve consecutive measurements. After the solutions have been measured, the stoppers of the vessels, greased with a good rubber lubricant (Stevens's rubber grease), are worked in by turning. The tissue is then prepared for the experiment. In order to equalise as nearly as possible the conditions of the two pieces of tissue, the portions to be used are suspended for 7 minutes in bicarbonate-containing medium through which oxygen containing 5 % carbon dioxide is passing and are then rinsed for a further 3 minutes in oxygenated bicarbonate-free salt solution. Portions are then weighed on a torsion balance after draining on filter-paper, the weights for each vessel being equal to the nearest mg., and are inserted in the medium in the spaces A. The vessels are attached to the manometer by the ground joints lubricated with rubber-grease, and placed in the bath. The gas stream (5 % CO₂ in O₂), passing first through a small water-containing gas-bubbler immersed in the bath and then through a cotton-wool plug, enters the apparatus through the upper tubes of the manometers and leaves through the stoppers of the vessels below the water-level in the bath. Extra gas-pressure should be applied by means of a screw-clip or tap on the safety device [Dickens and Greville, 1933, Fig. 3]. After the gas has passed for 10 minutes, during which the manometer has been continuously shaken, the extra pressure is released, the stoppers of the vessels turned and worked in until they will no longer move and the manometer shaken for a further 2 minutes with the vessels open to the air through the taps. The shaking is then interrupted, the vessels shut to the air and connected to the limbs, the menisci in the limbs being level and at the centre (150) mark. The right-hand tap is turned so that the limb is open to the air and the vessel closed: the acid is then tipped from the bulb into the medium in the right-hand vessel, the limb being temporarily taken out of the clips. After the right-hand vessel has been replaced, shaking is resumed. If glycolysis is to be measured, a simple manometer should have been placed in the bath at the same time as the differential manometer, and readings of both manometers are taken at intervals until the end of the experiment. When the differential manometer is used as a simple manometer, the two menisci are still kept to their appropriate marks, the position of the mark on the left-hand limb being read off as usual on the graduated scale on the right-hand limb.

At the end of the experimental period (usually 2 hours), during which 120–200 mm.³ oxygen should have been consumed, a final reading for use in calculation of glycolysis is taken, the meniscus in the left-hand limb is brought to the mark, the left-hand tap is turned so that the vessel is closed, and the acid is

tipped from the bulb. After shaking has been continued for a few minutes, both menisci are levelled at their marks, the limbs being open to the air. Both limbs are put in communication with their vessels and closed to the air, and 10 minutes after adding the acid a reading is taken, the constancy of which is checked 5 minutes later. This reading less $150 = H_1$. The permanganate is tipped into the iodide in both vessels and the manometer shaken until constancy of the reading shows that gas absorption is complete. This usually requires 35 minutes, but longer times, up to 1 hour, are occasionally necessary. The final reading less $150 = H_2$.

Calculation. Let h_{O_2} = pressure-change caused by consumption of oxygen and h_{CO_2} = pressure change due to respiratory carbon dioxide.

All pressures are reckoned algebraically in terms of mm. of Brodie fluid. With the procedure described, h_{O_2} is always negative, h_{CO_2} is always positive, whilst gas evolution in the left-hand (experimental) vessel always causes an increase in the reading.

After complete liberation of the bound carbon dioxide in both vessels, equal amounts of bound carbon dioxide having been present at the beginning of the experiment, any difference of pressure between the vessels is due only to the respiratory exchange during the experimental period.

$$\text{Hence} \quad H_1 = h_{O_2} + h_{CO_2} \quad \dots\dots(1).$$

After complete absorption of the carbon dioxide in the two vessels, the pressure difference is due to the oxygen consumed in the experimental vessel during the experimental period.

$$H_2 = h_{O_2} \quad \dots\dots(2).$$

Subtracting, $h_{CO_2} = H_1 - H_2$, and, as usual,

$$x_{O_2} = H_2 \cdot k_{O_2}; \quad x_{CO_2} = (H_1 - H_2) \cdot k_{CO_2},$$

where k_{O_2} and k_{CO_2} are the simple manometer vessel-constants [Warburg, 1926].

$$\text{Hence,} \quad \text{R.Q.} = - \frac{x_{CO_2}}{x_{O_2}} = \left(1 - \frac{H_1}{H_2}\right) \frac{k_{CO_2}}{k_{O_2}} \quad \dots\dots(3).$$

Now let h = pressure change read off on the instrument during the period in which it is used as a simple manometer, corrected for thermobarometer changes.

Then $h = h_{O_2} + h_{CO_2} + h_b$ where h_b = pressure-change due to alteration in the amount of bound CO_2 during the experimental period. By (1), $h_b = h - H_1$, so that

$$x_b = (h - H_1) k_{CO_2} \quad \dots\dots(4).$$

Table I.

Both vessels contained:

In A, 3 cc. bicarbonate solution, containing *ca.* 1700 mm.³ bound CO_2 .

In B, 0.3 cc. 2.5 N HCl.

In C, 2.0 cc. 43 % sodium iodide solution.

In D, 0.5 cc. 30 % sodium permanganate solution.

Filled with gas mixture containing 5 % carbon dioxide.

Pressure change on acidification of both vessels (corresponding to error in H_1):

+1, 0, +1, 0, +0.5, 0, -0.5 mm. Brodie fluid.

Pressure change on acidification followed by CO_2 absorption in both vessels (corresponding to error in H_2):

+1, +2, 0, 0.5, 0, 0 mm. Brodie fluid.

Table II.

Tissue	Moist wt. in each vessel (mg.)	Bicarbonate concentration (mM/litre)	Pressure difference (mm. Brodie fluid)	
			After acidification	After absorption
Rat testis	100	26	0	-1.5
Rat kidney	50	26	0	-1
Jensen rat sarcoma	95	26	-1	-3
"	100	26	-1	-1
"	100	34	-1.5	-1
"	100	34	-2	0

Protocols.

The protocols show results with examples of two types of tissue, (1) a normal tissue of known R.Q.=1, with little aerobic glycolysis, and (2) a tumour tissue with high aerobic glycolysis and low R.Q.

(1) Rat brain.

Thin slices of cerebral cortex; rinsed 5 mins. in bicarbonate-Ringer in stream of $O_2 + 5\% CO_2$, 5 mins. in salt solution in stream of O_2 , before experiment.

Gas mixture, O_2 with 5.46 % CO_2 . Bicarbonate content of Ringer 530 mm.³ CO_2 /cc.

Glucose 0.2 %. 37.2°. p_H 7.4.

Differential manometer filled with Brodie fluid: vessel vols. 23.23 cc. (both).

Contents of vessels:

A, 3.0 cc. bicarbonate-Ringer-glucose solution + tissue.

B, 0.25 cc. 2.5 N HCl.

C, 2.0 cc. 43 % sodium iodide solution.

D, 0.5 cc. 30 % sodium permanganate solution.

Vol. fluid 5.75 cc. Vessel $\begin{cases} k_{O_2} = 1.55 \\ \text{consts. } k_{CO_2} = 1.84. \end{cases}$

Experiment:

Differential manometer. Pressure readings (mm. Brodie fluid).

Time (mins.)	
0	150
2	150
120	156
130	136.5
135	137
170	70
175	69.5
185	70

Acidify closed R.H. vessel.

$h = +3$ (corrected for thermobarometer).

Close L.H. vessel and acidify. Level at 150. Open both vessels to manometer.

$H_1 = -13$. Add $NaMnO_4$ to NaI, both vessels.

$H_2 = -80$.

Weight of tissue $\begin{cases} \text{wet (L.H.) 60 mg.; (R.H.) 60 mg.} \\ \text{dry (L.H.) 5.5 mg.; (R.H.) 6.3 mg.} \end{cases}$

Calculation:

Time of experiment: 2 hrs. 0 min.

$$H_2 = h_{O_2} = -80 \text{ mm. } x_{O_2} = -124 \text{ mm.}^3$$

$$h_{CO_2} = H_1 - H_2 = +67 \text{ mm. } x_{CO_2} = +123 \text{ mm.}^3$$

$$h_b = h - H_1 = +16 \text{ mm. } x_b = +29 \text{ mm.}^3$$

Whence $Q_{O_2} = -11.3$ (mm.³ O_2 per mg. dry tissue per hour).

$$Q_M^{O_2} = +2.6 \text{ (mm.}^3 \text{ acid produced per mg. dry tissue per hour).}$$

$$\text{R.Q.} = 0.99.$$

(2) *Jensen rat sarcoma.*

11-day tumour; thin slices; pre-treatment, vessels, vessel-constants, gas mixture and solutions, as for brain (1), except that bicarbonate content of Ringer solution = 666 mm.³/cc.; hence p_H (initial) = 7.5; (final) = 7.3. 37.2°.

*Experiment:**Differential manometer.*

Time (mins.)		
0	Level at 150 mm. and acidify closed R.H. vessel.	
30	+ 20.5	Pressure changes in 30-min. periods corrected for thermobarometer.
60	+ 22.5	
90	+ 25	
120	+ 27	
150	+ 27	
<hr/>		
	$h = +122$	Close L.H. vessel and acidify. Level menisci at 150 mm. Open both vessels to manometer.
Pressure readings		
160	109	
165	110	
<hr/>		
210	36	$H_1 = -40$. Add NaMnO ₄ to NaI, both vessels.
220	32.5	
235	32.5	$H_2 = -117.5$.

Weights of tissue { wet (L.H.) 55 mg.; (R.H.) 55 mg.
dry (L.H.) 5.3 mg.; (R.H.) 6.2 mg.

Calculation:

Time of experiment 2 hrs. 30 mins.

$$H_2 = h_{O_2} = -117.5 \text{ mm. } x_{O_2} = -182 \text{ mm.}^3$$

$$h_{CO_2} = H_1 - H_2 = +77.5 \text{ mm. } x_{CO_2} = +142.5 \text{ mm.}^3$$

$$h_b = h - H = +162 \text{ mm. } x_b = +298 \text{ mm.}^3$$

Whence

$$Q_{O_2} = -13.7.$$

$$Q_M^{O_2} = +22.4.$$

$$R.Q. = 0.78.$$

Simultaneously made experiments on slices of the same tumour suspended in 1.5 cc. bicarbonate-Ringer by Dickens and Šimer [1931] method gave $R.Q. = 0.81$, $Q_{O_2} = -12.7$; whilst a determination of aerobic glycolysis by Warburg's 2-vessel method gave $Q_M^{O_2} = +22.3$, if the value of $R.Q.$ found by the constant-volume differential manometer experiment given above be assumed for the calculation.

Notes.

(1) *Absorption of carbon dioxide.* The production of an absorbent for carbon dioxide by the mixing of two solutions, such as NaI and KMnO₄, avoids the necessity of having taps for the introduction of alkali into the vessel during the experiment. The quantities of the reagents used for carbon dioxide absorption by Dickens and Šimer [1931] are insufficient for the present method; more concentrated solutions are used, and it is necessary to use the more soluble sodium permanganate. The pressure change accompanying the mixing of iodide and permanganate which has no relation to CO₂ absorption may become large with concentrated solutions. It is probably due to alterations either of vapour pressure or of volume. The pressure changes observed on adding 30 % permanganate to sodium iodide solutions of various concentrations are shown in Fig. 2, from which it is seen that by choosing suitable concentrations the pressure change on mixing strong solutions may be reduced below that found with the original dilute solutions. We have therefore used in all experiments 30 % NaMnO₄, 3H₂O and 43 % NaI, 2H₂O, 0.5 cc. of the former being added to 2 cc. of the latter. Carbon dioxide absorption by these quantities is rapid and complete. Since they were found to absorb completely 6.4 cc. carbon dioxide, *i.e.* 25 % of the vessel volume and twice as much carbon dioxide as is likely to be in the vessel during a $R.Q.$ determination, it is clear that an ample excess of alkali is produced.

(2) *Accuracy.* In the absence of tissue, errors may be introduced by inaccurate measurement of the bicarbonate solutions and unequal filling of the two vessels with gas. The magnitude of the errors occurring in absence of tissue with careful measurement and technique as described is shown by Table I. When equal wet weights of tissue are inserted into the two vessels, the accuracy is limited by the agreement in their initial bicarbonate contents and in their glycolyses during the preliminary period. In the control experiments in Table II amounts of tissue suitable for r.q. determinations were prepared and the vessels filled by the technique described, but 2 minutes

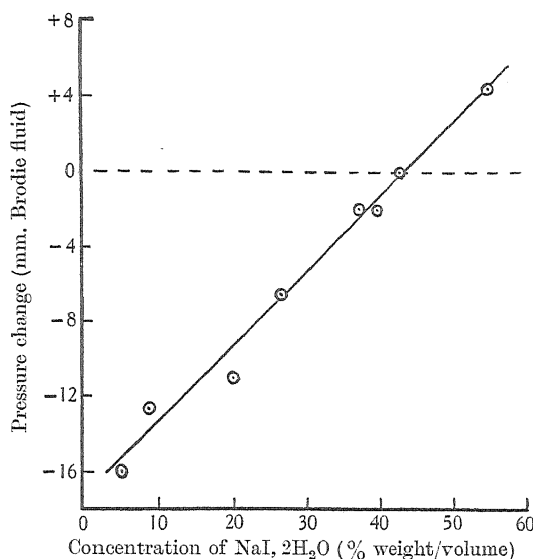


Fig. 2. Pressure changes on adding 0.5 cc. 30% NaMnO_4 , $3\text{H}_2\text{O}$ to 2 cc. of sodium iodide solutions of various concentrations; 37.3° ; air in gas space. 0.2 cc. N NaOH in vessel as CO_2 absorbent. Permanganate solution contained $N/400$ H_2SO_4 , iodide contained $N/100$ H_2SO_4 .

after stopping the gas stream the acid was tipped from the bulbs into both vessels. After a steady reading had been obtained, the carbon dioxide was absorbed in both vessels. The figures in the fourth column correspond to errors in H_1 , those in the fifth to errors in H_2 . In the last two experiments the strong bicarbonate-Ringer solution now used for tumour experiments (see Protocol) was taken. H_2 is always much larger than H_1 , and errors of a few mm. in H_2 have a negligible effect on the r.q. The possible error in the latter, governed by the error in H_1 , with a respiration corresponding to 100 mm. Brodie fluid, is $\pm 2.5\%$. This is equal to the error of the Dickens and Šimer method [1931]; but it is to be noted that in the present method the necessary respiration is only one-half and the bicarbonate reserve over twice that obtaining in the earlier one.

(3) Any oxygen uptake or carbon dioxide production during the experimental period by the tissue in the vessel in which the acid is added from the bulb at the beginning of the experiment will lead to faulty values of x_{O_2} and x_{CO_2} . The significance of oxygen uptake by acidified tissue in the measurement of r.q. is discussed by Needham [1932, 1, 2]. We have found, however, that various types of tissue, when acidified under conditions comparable with those of the acidified tissue in the present method, show an oxygen uptake always less than 1% of that of the non-acidified tissue. Further, in absence of carbon dioxide absorbent, such acidified tissue causes no pressure change. It is therefore concluded that the presence of acidified tissue in this method has a negligible effect on the observed value of the r.q.

If at the beginning of the experiment, the metabolism of the tissue in one vessel could be immediately and completely stopped without altering the acid-base equilibrium, the presence of acidified tissue during the experimental period would be avoided, and also there would be no need

for a thermobarometer to assist in the measurement of h . The method would thereby be improved, but no suitable agent for killing the tissue has yet been found.

(4) This method has been developed during the last two years independently of that described recently by Dixon and Keilin [1933], which is essentially an application of the principle of the Dickens and Šimer [1931] method to the Barcroft manometer. Not only has the application of the constant-volume differential manometer, in preference to the Barcroft instrument, made possible a method which combines an increased sensitivity with a larger bicarbonate reserve, but also the flexibility of the rubber connection between the two limbs allows the use of rigidly fixed vessels without the necessity for ground-in bulbs and for the introduction of extraneous fluids by taps attached to the vessel. It is our experience that only stoppers which have been worked in until they will no longer turn are consistently capable of withstanding without danger of leak the large pressure differences involved in the determination of a respiratory quotient.

SUMMARY.

The constant-volume differential manometer previously described by the authors has been applied to the measurement of respiratory quotient, respiration and glycolysis in bicarbonate media, with the help of the principle used by Dickens and Šimer [1931] in the earlier method for this purpose. Advantages of the new method are:

1. It is not necessary to use dense manometric fluids.
2. The sensitivity is increased above that of the previous method, so that for the same accuracy less tissue and a shorter experimental period may be used.
3. At the same time the bicarbonate reserve in the vessels may be greatly increased. This enables accurate measurement of the aerobic acid production of highly glycolysing tissue, for which the previous method gave low values.
4. The conditions for measurement of glycolysis are comparable with those in the Warburg two-vessel method; but the new method, like the other two described in this series, has the great advantage that the metabolism is measured on a single piece of tissue.

We wish to make acknowledgments to the Halley-Stewart Trust Fund for a scholarship held by one of us (G. D. G.).

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CCI. THE ABSORPTION OF *n*-HEXADECANE FROM THE ALIMENTARY TRACT OF THE RAT.

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(Received August 26th, 1933.)

THE degree to which unsaponifiable substances are absorbed from the alimentary tract has assumed considerable importance in the last few years on account of the biological significance of the fat-soluble vitamins and carotene. In previous papers the absorption of squalene, phytol, oleyl and cetyl alcohols, cholesterol and a medicinal liquid paraffin has been investigated [Channon, 1926; Channon and Collinson, 1928; 1929]. In the last case, it was found that the liquid paraffin was absorbed to some extent by the rat. Further, from consideration of the cholesterol content of the liver unsaponifiable fractions and of the iodine values of the non-sterol portion of those fractions, it was deduced that the liquid paraffin was present in the liver. By further experiments on pigs, it was found possible chemically to isolate the paraffin from the liver of that animal. These results were of interest because firstly they demonstrated the absorption of hydrocarbons from the alimentary tract to a limited extent at least, even though previous investigations had led to the current view that such hydrocarbons were not absorbed, and secondly they focused attention on the apparent rôle of the liver in storing unsaponifiable substances generally. Demonstration of the former fact raised the question as to the mechanism whereby such inert substances as hydrocarbons are absorbed. Since chemical investigation of the liquid paraffin used showed that it consisted of a mixture of polycyclic hydrocarbons, it was felt that the positive results obtained for its absorption, which seemed at variance with previous work, might be attributable to its particular chemical nature. For this reason, it was deemed necessary to carry out further work using a synthetic normal hydrocarbon.

EXPERIMENTAL.

Preparation of hexadecane.

n-Hexadecane was chosen for this work because it can be prepared readily from palmitic acid and secondly, the fact that it is liquid at room temperature (M.P. 18°) should facilitate its absorption. Pure ethyl palmitate was prepared either from palm oil or commercial palmitic acid. In the main preparation 348 g. of ester dissolved in the minimum volume of warm alcohol (which had been previously distilled after dissolution in it of 2 % of sodium and then boiled for a day with metallic calcium and again distilled) were slowly run on to 209 g. of sodium at 130°. The cetyl alcohol isolated weighed 280 g. (94 %

of theory). The alcohol was converted into the iodide by means of red phosphorus and iodine and the iodide converted into *n*-hexadecane by treatment with zinc dust in glacial acetic acid into which hydrochloric acid gas was occasionally bubbled for a few minutes. The hexadecane was then distilled. The first preparation (B.P. 287°, M.P. 18°) gave C 84.48, H 14.68 % (calc. C 84.85, H 15.15 %) and this was used for exp. I. For exp. II, part of this material was again distilled and the product used gave C 84.60, H 15.1 %. For exp. III, the large preparation after purification with concentrated sulphuric acid at 130° gave C 84.81, H 15.15 %.

Animal experiments.

In each of exps. I and II, two groups of rats, containing the same number of animals of each sex, were fed on a synthetic diet (caseinogen 20, butter 10, starch 60, marmite 5, salt mixture 5) for 21 and 20 days respectively. The experimental groups received in addition, immediately before feeding, doses of hydrocarbon delivered into the mouth from a micro-burette, the dose being gradually increased from 0.035 to 0.12 cc. daily. The animals were then killed by chloroform after being without food for 24 hours. Their livers were removed and the unsaponifiable fractions prepared as described by Channon [1926].

The faeces had been collected twice weekly and stored under absolute alcohol and at the close of the experiments the cages and trays were rinsed with ether, which was added to the main extracts. The faeces from each batch of rats were then boiled separately with a litre of absolute alcohol. This alcohol extraction was repeated three times and followed by three extractions with ether under reflux. The finely powdered residues were then further treated with ether for 12 hours in a Soxhlet extractor. After evaporation to dryness of the combined alcohol-ether extracts, the material was saponified with strong alcoholic potassium hydroxide and the unsaponified material extracted by ether. After a second saponification of this material, the faecal unsaponifiable fraction was obtained. Cholesterol was determined by the digitonin method and the iodine value by the Rosenmund and Kuhnemann [1923] methods on the unsaponifiable fractions from both livers and faeces.

The results are recorded in Table I.

Exps. I and II. Table I.

	I		II	
	20		21	
	12		14	
	Animals receiving the control diet		Animals receiving control diet <i>plus</i> hexadecane	
	I	II	I	II
The unsaponifiable matter of the livers				
No. of animals	9	10	9	10
Wt. of livers (g.)	69.4	77.2	79.9	82.3
(a) Wt. of unsap. fraction (g.)	0.226	0.320	0.238	0.3835
Wt. of sterol in (a) (g.)	0.162	0.150	0.190	0.1788
(b) Wt. of non-sterol fraction of (a) (g.)	0.064	0.170	0.048	0.2047
Iodine value of (a)	94.7	87.3	89.1	90.7
The unsaponifiable matter from the faeces				
(a) Wt. of unsap. fraction (g.)	2.60	2.26	5.80	6.40
Iodine value of fraction	69.0	62.6	32.8	30.1
Wt. of sterol in unsap. fraction (g.)	0.78	0.72	1.00	1.02
Wt. of non-sterol fraction of (a) (g.)	1.82	1.54	4.80	5.62

The results are rendered more lucid by the figures in Table II in which are recorded the yields of unsaponifiable material and sterol per 100 g. of liver, together with the iodine values of the non-sterol fraction calculated from the cholesterol content.

Table II. *Yields of unsaponifiable matter and cholesterol mg./100 g. of liver.*

<i>Exp. I</i>	Unsap. matter	Sterol	Non-sterol	Iodine value of non-sterol fraction
Control	325	233	92	169
Control + hexadecane	298	238	60	181
<i>Exp. II</i>				
Control	415	194	221	106
Control + hexadecane	466	217	249	111

The mean values calculated from 11 control groups of animals in results reported by Channon [1926] and Channon and Collinson [1928] in similar experiments are: unsap. matter 414, cholesterol 296, non-sterol 118 mg. per 100 g. liver, and the figures recorded in Table II all lie within the range of normal variation in those 11 experiments.

Thus the administration of the hydrocarbon has had no significant effect on the yield of the unsaponifiable material of the livers, or on its sterol content. Further, the iodine values of the non-sterol part of the unsaponifiable fractions show no significant difference between the control and experimental animals. With these figures may be contrasted the results of the two experiments of Channon and Collinson [1929] with the polycyclic hydrocarbon mixture. As a mean value for two experiments, these authors found the significant result that in the control animals the material other than sterol in the unsaponifiable fraction constituted but 22.7 % of the whole and had an iodine value of 118.8, as against 56.2 % and 30.8 for the corresponding figures for the animals which received the polycyclic paraffin. It is to be noted however that they too observed no increase in the actual amount of the unsaponifiable matter in the livers.

The unsaponifiable matter of the faeces.

From the figures recorded in Table I on the unsaponifiable fraction prepared from the faeces, certain deductions may be made. In exp. I the weight of the non-sterol fraction in the control group is 1.82 g., and in the faeces of the animals which received the hydrocarbon, 4.80 g. The difference between these two values, namely 2.98 g., gives an approximate value for the amount of the hydrocarbon excreted. Further, an approximate check on this value may be obtained from calculations made from the iodine values of the unsaponifiable fractions and their sterol content, provided it be remembered that the use of the iodine value of cholesterol, 65.8, for the mixture of sterols present in the faeces is arbitrary. Calculation on this basis shows in the control group of exp. I that the fraction of the unsaponifiable matter which is not sterol, 1.82 g., has an iodine value of 70.4, whilst the corresponding fraction for the animals fed with hexadecane, 4.80 g., has an iodine value of 25.9. If the increased amount of the faecal unsaponifiable matter in the latter group is due to the presence of hexadecane, of iodine value zero, and if that part of the unsaponifiable matter which is neither sterol nor hydrocarbon has the same iodine value as the corresponding fraction from the control group, namely 70.4, the amount of the latter may be calculated. The figure so obtained for the amount of hexadecane in the

faecal material is $4.80 - 1.75 = 3.05$ g. which agrees well with the figure obtained directly by difference between the weights of the non-sterol fractions, *i.e.* 2.98 g. Therefore in exp. I, of 12 g. of hexadecane administered, 3 g. only have been excreted. Similar calculations may be applied to exp. II, and the combined results are set out in Table III.

Table III. *Excretion of hexadecane in the faeces.*

	Animals receiving control diet		Animals receiving control diet <i>plus</i> hexadecane	
	Exp. I	Exp. II	Exp. I	Exp. II
(a) Wt. of non-sterol fraction (g.)	1.82	1.54	4.80	5.62
(b) Iodine value of non-sterol fraction (calc.)	70.4	61.2	26.3	22.3
(c) Wt. of material other than hydrocarbon in (a) (g.) (calc. from (b))	—	—	1.75	2.05
(d) Wt. of hydrocarbon (g.):				
(1) By difference from (a)	$4.80 - 1.82 = 2.98$		$5.62 - 1.54 = 4.08$	
(2) By calc. from (b)	$4.80 - 1.75 = 3.05$		$5.62 - 2.05 = 3.57$	
(e) Wt. of hexadecane (g.) (Mean values of (d) 1 and (d) 2)	Exp. I	Exp. II		
Hexadecane administered (g.)	3.00	3.83		
Hexadecane not recovered (g.)	12.00	14.00		
	9.00	10.17		

In the two experiments therefore 9.0 and 10.17 g. of the hexadecane administered were not recovered in the faeces. This corresponds with an absorption of 50 and 49 mg. per rat per day respectively. Hence, although there has been no alteration in the amount or character of the unsaponifiable matter of the livers, a definite amount of the hydrocarbon has been absorbed. In view of the fact that Channon [1926] isolated squalene from the livers of rats which had received squalene in their diet, and that Channon and Collinson isolated the polycyclic hydrocarbon from the liver of a pig which had received liquid paraffin, the failure of the hydrocarbon to appear in the liver is unexpected. Since the liver seems to fulfil the rôle of storing unsaponifiable substances, the absence of hexadecane suggested that the 19 g. absorbed by 19 rats in 21 days may have been completely oxidised or less probably stored in part elsewhere. It was accordingly decided to carry out a further experiment more closely akin to the conditions of that of Channon and Collinson [1929] and at the end to attempt to determine whether the hydrocarbon were present not only in the livers but also in the carcasses. In this experiment, the hydrocarbon was incorporated in a similar diet to the extent of 4 % for 15 days which was increased to 6 % for a further 9 days, the percentage of starch being correspondingly reduced. Every care was taken to keep the experiment as quantitative as possible and the following method was adopted for incorporating the hexadecane in the diet: one-half the butter required for a given quantity of diet was melted and run in to a weighed amount of hydrocarbon in another vessel. After mixing, the butter-hydrocarbon mixture was incorporated in the dry diet, the remainder of the butter being used successively to rinse out the vessel in which the hexadecane and butter had been mixed.

The unsaponifiable fractions of the livers and faeces were prepared as in the previous experiments, and after removal of the entire alimentary tract the carcasses were also worked up. They were warmed on a water-bath with 5 % aqueous sodium hydroxide until digestion was complete, when the fat was exhaustively extracted by ether. The fat was then resaponified with alcoholic

potash and the unsaponified material obtained. In view of the large amount of fat present prior to the second saponification, the resultant product was again submitted to saponification with alcoholic sodium ethoxide. The results of exp. III are set out in Table IV.

Table IV.

Exp. III.

Duration of experiment 25 days. Hexadecane administered 149 g.

	Animals receiving the control diet	Animals receiving the control diet plus hexadecane
The unsaponifiable matter of the livers		
No. of animals	13	13
Wt. of livers (g.)	107.2	111.0
Wt. of unsap. fraction (g.)	0.3403	0.3276
Wt. of sterol in unsap. fraction (g.)	0.2327	0.2402
Iodine value of unsap. fraction	83.3	65.8
Wt. of material other than sterol (g.)	0.1076	0.0874
The unsaponifiable matter of the carcasses after removal of the alimentary tract and liver		
Wt. of unsap. fraction (g.)	5.01	8.04
Wt. of sterol in unsap. fraction (g.)	2.68 (53.5 %)	3.63 (45.1 %)
Iodine value of unsap. fraction	73.8	58.2
Wt. of material other than sterol (g.)	2.33	4.41
The unsaponifiable matter of the faeces		
Wt. of unsap. fraction (g.)	3.90	118.3
Iodine value of unsap. fraction	53.7	9.4

The results calculated on a percentage of the weight of liver are recorded in Table V.

Table V. *Yields of unsaponifiable matter and cholesterol mg./100 g. of liver.*

	Unsap. matter	Sterol	Non-sterol	Iodine value of non-sterol
Control diet	317	217	100	122
Control diet + hydrocarbon	295	216	79	66

These figures also show no increase in the amount of the unsaponifiable matter in the livers of the experimental group although the iodine value of the non-sterol fraction is 66 compared with 122 for that of the control animals, a result similar to that of Channon and Collinson, previously mentioned. The amount of hydrocarbon in the faeces, about 114 g., leaves 35 g. unaccounted for. This corresponds to a daily absorption per rat of 108 mg. an amount rather more than twice as great as was obtained in exps. I and II, a result to be anticipated because in those experiments the animals received the hexadecane in drops before being fed in the morning, while in exp. III the considerably greater amount of hydrocarbon administered was dissolved in the butter before admixture with the rest of the diet.

On the other hand, although increase in the amount of unsaponifiable matter of the livers has occurred, study of the figures recorded in Table IV reveals the fact that the amount of unsaponifiable matter yielded by the carcasses of the experimental animals, 8.04 g., is some 60 % greater than that of those of the control group, 5.01 g.

Further the non-sterol fraction present is 4.41 g. compared with 2.33 g. of the control group. Assuming that the increase is due to the presence of hexadecane only, simple subtraction suggests the presence of 2.08 g. of that substance.

Further, if the iodine value of the material which is not sterol or hydrocarbon in the unsaponifiable fraction from the animals which received hexadecane is the same as that of the non-sterol material from the control group, calculation shows that the former must contain 1.65 g. of a saturated substance. This figure is in fair agreement with that obtained by simple subtraction, 2.08 g. The presumptive evidence of the presence of the hydrocarbon in the carcasses was strengthened by the semi-liquid appearance of the unsaponifiable fraction from the experimental group and its low acetyl value, but it was not finally confirmed by chemical isolation of the hexadecane. We believe, however, that the evidence is sufficiently convincing to enable us to say that from 1.5 to 2 g. of hexadecane or some other saturated substance derived from it has been stored in the tissues other than the liver as a result of feeding. Thus of 149 g. of hexadecane administered, about 114 g. has been excreted in the faeces, and of the remaining 35 g., about 1.85 g. has appeared in the tissues, leaving about 33 g. unaccounted for.

DISCUSSION.

The degree to which the hexadecane has been absorbed needs to be assessed with caution from these results. In exps. I and II the amount of hydrocarbon not recovered in the faeces corresponds to 49 and 50 mg. per rat per day respectively and in exp. III to 108 mg. per day. These figures are open to two possible sources of error: firstly that the accuracy of measurement as determined by the intake and excretion method is limited for the reasons discussed in a previous paper [Channon and Collinson, 1928] and secondly it is not impossible that part of the hexadecane may have been attacked by bacteria in the large intestine, since, as is mentioned later, certain moulds at least are able to utilise higher *n*-hydrocarbons as a sole source of carbon. We may perhaps dismiss the latter possibility as one unlikely seriously to impair the results on account of the slowness with which such an action is likely to take place, while the former should not in any case render the results markedly inaccurate. If therefore the results are accepted at their face value, a number of deductions may be made.

(1) That *n*-hexadecane is absorbed from the alimentary tract of the rat to the extent of between 50 and 100 mg. per day.

This result is essentially similar to those previously obtained with squalene [Channon, 1926] and with a liquid paraffin which was proved to be a mixture of polycyclic hydrocarbons [Channon and Collinson, 1929] in that absorption is definite although the degree of absorption is very limited. It is to be noted that previous experiments suggest that squalene is absorbed even to a less extent than is hexadecane. So far as appears at present therefore the chemical nature of the hydrocarbon has little effect on its degree of absorption which is possibly conditioned by its solubility in bile salt solutions of fatty acids.

(2) In exp. III, in which the unsaponifiable matter from the entire bodies of the animals was prepared, of 35 g. of hexadecane not recovered in the faeces, only about 1.5 g. were present in the tissues, and hence 13 rats have apparently metabolised 33.5 g. in 25 days. Such a finding that the animal appears to possess the power to metabolise a normal aliphatic hydrocarbon is an unexpected one. On the other hand the work of Tausson [1925] and Hopkins and Chibnall [1932] has shown that certain moulds can utilise higher paraffins as a sole source of carbon, while Tausz and Peter [1929] showed that certain soil bacteria possess similar powers. The utilisation of higher normal hydrocarbons by moulds shows that these substances, even though possessing no polar group, are not immune from attack at least by this form of living matter, and hence the disappearance

of some 33 g. of hexadecane in our experiments on the rat may be less surprising than appears at first sight.

(3) The third point worthy of note is that the absorption of hydrocarbon has not resulted in an increase in the percentage of the unsaponifiable matter in the liver. This result is the same as was obtained by Channon and Collinson [1929] when they administered a liquid paraffin to rats, where the percentage of unsaponifiable matter did not rise although the iodine value of the non-sterol fraction was modified in a similar way to that in exp. III of this paper. It was this finding which led these workers to continue the work on pigs from the liver of which the hydrocarbon was isolated. On the other hand, when squalene was administered to rats Channon [1926], the liver unsaponifiable matter rose to about 1 %. It is difficult to suggest why the effects of the administration of squalene which is apparently less well absorbed should cause such a marked rise in the liver unsaponifiable matter, when that of hexadecane causes no rise whatever. Similarly, of the alcohols previously investigated in this experiment phytol, oleyl and cetyl alcohols and cholesterol only the last caused a significant rise in the liver unsaponifiable matter.

(4) The failure of the hydrocarbon to raise the liver unsaponifiable matter significantly is the more surprising in view of the presence of the hydrocarbon in other tissues of the animals, presumably the fat of adipose tissues. During recent years work on the fat-soluble vitamins and the investigations which have been carried out on other substances of peculiar biochemical interest which occur in various fish-liver oils (batyl, selachyl, chimyl and other alcohols, squalene and other hydrocarbons) has tended to focus attention on the liver as the tissue where unsaponifiable substances are generally to be found. This is in part because other tissues, so far as the slight extent to which they have been investigated shows, contain unsaponifiable substances in relatively insignificant amount only. Further, cholesterol is the main constituent of this fraction. Hence it may appear surprising that the hydrocarbon should not be obviously present in the liver. On the other hand, presumably the capacity of the liver for unsaponifiable substances generally must be limited by their nature and therefore it would perhaps be that such a saturated substance as a hydrocarbon would find its way into the fat depots, if it were not readily oxidised.

SUMMARY.

1. Synthetic *n*-hexadecane has been administered to two groups of 9 and 10 rats for periods of 21 days respectively and the degree of absorption measured by the preparation and analysis of the unsaponifiable fractions of the faeces of these and the control groups of animals.

2. The results indicate an absorption by the rat of 50 mg. of hexadecane daily. This absorption had no effect on the amount of the unsaponifiable matter of the livers, nor on their cholesterol content, nor on the iodine value of the non-sterol fraction.

3. In a third experiment 149 g. of synthetic *n*-hexadecane were administered to 13 rats for 25 days, being incorporated in the diet to the extent of 5 %.

4. The degree of absorption measured by the intake and excretion method corresponded to each rat having absorbed 108 mg. per day and resulted in the iodine value of the non-sterol fraction of the unsaponifiable matter of the livers falling from the control value of 122 to 66.

5. After removal of the livers and alimentary tracts, the carcasses of the animals were examined for the hydrocarbon, and of the 35 g. of the latter not

recovered from the faeces, evidence was obtained of the presence of 1.85 g. in the tissues.

6. The disappearance of the remaining 33.15 g. suggests that the animal organism can metabolise normal aliphatic hydrocarbons.

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CCII. ON THE THERMOSTABILITY OF PROLAN.

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(Received July 21st, 1933.)

MANY authors have confirmed Zondek and Aschheim's [1928; 1929] original experiments on the thermolability of the ovary-stimulating substance found by them in the anterior pituitary lobe and in the urine of pregnant women². All the observations, however, appear to have been made on material heated in aqueous solution, and the possibility that heating the substance in the dry state might give different results has been overlooked. In attempting high vacuum distillation of the active substance from a dried crude extract of urine, we were led to examine the activity of residues subjected to temperatures of 80°, 100° and 150° for an hour in a high vacuum. It has been found that the active substance of urine of pregnancy is not readily destroyed at 100° when heated in the form of a dry powder, although we have confirmed the fact that heating in the presence of water results in complete and rapid destruction.

Methods and material.

Prolan extract. The same urine extract was used throughout the experiments; it was stored at -2° as a dry powder, under which conditions it remains indefinitely stable. The extract was crude; 10 mg. caused ovulation in 39 out of 40, and 5 mg. in 33 out of 65 oestrous rabbits [Parkes and White, 1933]. 10 mg. have therefore been considered as the amount required to cause ovulation with reasonable certainty. The heated samples were tested in the same way, by intravenous injection followed next day by a sterile laparotomy under ether to ascertain if ovulation had occurred.

Heating in dry condition. The samples heated *in vacuo* were spread on the inside of a thin-walled glass vessel, which was then evacuated by a two-stage mercury vapour pump connected through a trap cooled with liquid oxygen. The vacuum attained was higher, probably much higher, than 10⁻³ mm. Hg. The evacuated vessel was heated in a suitable liquid bath.

Samples heated in oxygen or nitrogen were placed in an ampoule, which was then alternately evacuated and filled four times with the required gas dried by passage over P₂O₅. The ampoule was sealed off after the final admission of dry gas.

Heating in aqueous solution. For obtaining an oxygen-free solution, the sample was weighed into an ampoule from which air was displaced by a current of nitrogen. 10 cc. of distilled water, boiled and cooled in a current of nitrogen,

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² To material from this latter source Zondek and Aschheim have given the name "prolan," which is used here for lack of a better one.

were added. During the heating a slow stream of nitrogen was passed through the solution.

In the converse experiments, oxygen was bubbled through the solution during the heating.

Effect of heating in aqueous solution.

Confirmatory experiments on the thermolability of prolan tested in aqueous solution were carried out as shown in Table I. The results show that:

(a) Very brief heating to 100° in water causes destruction. Our actual experimental figures show a loss of not less than 90 % after 5 minutes. Tests on material heated for 1 hour were carried rather further and indicate a loss of not less than 98 %. Destruction is probably complete in both cases.

Table I. *Effect of heating prolan in aqueous solution.*

No. of exp.	Temperature (°)	Duration of heating (hrs.)	Atmosphere	Amount tested (mg.)	Result
3 a	100	1	Oxygen	25	-
1 c	100	1	Nitrogen	30	-
1 d	100	1	Nitrogen	30	-
3 c	100	1	Oxygen	100	-
3 d	100	1	Nitrogen	500	-
6 c	100	$\frac{1}{2}$	Nitrogen	100	-
7 b	100	$\frac{1}{2}$	Nitrogen	250	-
7 a	100	$\frac{1}{2}$	Nitrogen	100	-
9 a	60	1	Nitrogen	10	+
9 b	60	1	Nitrogen	10	-
10 a	60	1	Nitrogen	10	+
10 b	60	1	Nitrogen	10	-
8 a	60	1	Air	20	+
8 b	60	1	Air	20	+
7 c	60	1	Nitrogen	50	-
8 c	60	1	Air	50	+
8 d	60	1	Air	50	+
6 a	60	1	Nitrogen	100	+
8 f	60	1	Air	100	+

(b) The destructive action does not appear to be influenced by the atmosphere in which the solution is heated.

(c) Heating to 60° in water for 1 hour is less destructive. At least 50 % of the activity survives this treatment.

Table II. *Effect of heating prolan in the dry state.*

No. of exp.	Temperature (°)	Duration of heating (hrs.)	Atmosphere	Amount tested (mg.)	Result
GS 7 R	80	$\frac{1}{2}$	Vac.	20	+
GS 9 R	100	1	Vac.	20	+
GS 9 R	100	1	Vac.	10	+
1 a	100	1	Nitrogen	10	+
2 b	100	1	Oxygen	10	+
2 c	100	1	Oxygen	10	-
2 d	100	1	Oxygen	10	+
4 b	100	1	Oxygen	10	+
4 c	100	1	Oxygen	10	+
4 d	100	1	Oxygen	10	+
4 e	100	1	Oxygen	10	+
GS 11 R	100	1	Vac.	5	-
GS 11 R	100	3	Vac.	10	+
GS 12 R	100	3	Vac.	10	+
6 b	150	1	Nitrogen	100	+
8 c	150	1	Nitrogen	50	+
GS 13 R	150	1	Vac.	20	-

Effect of heating in the dry condition.

The results obtained by heating the dry powder were in striking contrast to those given by heating aqueous solutions. The experiments are summarised in Table II which shows that 10 mg. of the dry powder heated to 100° for 1 hour or more gave a positive response in 10 out of 11 experiments. Comparing this response with the known activity of the unheated powder, it is evident that resistance to the treatment must have been almost if not quite complete. Even heating to 150° for 1 hour failed to destroy all activity, five times the usual dose being active after such treatment.

DISCUSSION.

The experiments described above demonstrate conclusively that whereas the ovulation-producing substance of urine of pregnancy is readily destroyed by heat in the presence of water, it is remarkably thermostable in the dry state. This difference in stability to heat confirms the suggestion that the destructive action is hydrolytic in nature [Marshall, 1932] but, unfortunately, it is not easy to see what use can be made of the property in the purification of extracts.

On the other hand, one important practical use can be made of the findings recorded above, *i.e.* in sterilising the extract. Adequate sterilisation for injection has hitherto been a serious difficulty in the administration of urine extracts. Bacterial filters suffer from two drawbacks: (*a*) the active principle is absorbed on the asbestos unless the p_H is carefully adjusted, and (*b*) the final product is necessarily a solution, in which form the substance is not stable. By taking advantage of the thermostability in the dry state, it will be easy to enclose within one ampoule the required amount of sterile powder and sterile water, with a suitable arrangement for mixing when required for injection¹.

SUMMARY.

(1) The ovulation-producing substance of urine of pregnancy is readily destroyed by heating in aqueous solution.

(2) The substance is, however, remarkably thermostable in the dry state; exposure to 100° for 1 hour or more causes no detectable loss of activity.

(3) The exclusion of oxygen does not appear to affect the stability either in aqueous solution or the dry state.

¹ Ampoules of this type are already used by pharmaceutical firms for other substances.

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CCIII. THE VITAMINS PRESENT IN OX-SERUM. I.

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(Received August 9th, 1933.)

IN the investigation described below the presence of vitamins A and B in ox-serum was sought for by means of biological tests.

Sherman and Boynton [1925] have already shown that rat-blood contains vitamin A.

Its presence in serum is a matter of inference, rather than of direct evidence, from the experiments of Euler and Virgow [1932], who showed that the carotene isolated from serum possesses vitamin A activity.

Vitamin B₁ was found absent from blood [Kollath, 1930] while vitamin B₂ was found to be present [Hoagland, 1923].

TECHNIQUE.

The experiments were carried out with white rats, all being pure bred Wistar stock and reared at the laboratory. The young were put on to the experimental diets when 3-4 weeks old and weighing between 30 and 40 g.

Four diets were used in the experiments having the following composition: the quantities given are the daily rations per rat in g. (or cc. in the case of liquids).

	Diet 1	Diet 2	Diet 3	Diet 4
Caseinogen	3	2½	3	2½
Hardened cottonseed oil	2	2	2	2
Rice starch	10	10	10	10
Lemon juice	1	1	1	1
Salt mixture	1	1	1	1
Dried yeast	1	1	1	0
Irradiated cholesterol	1/200	1/200	1/200	1/200
Cod-liver oil	1/20	0	0	1/20
Ox-serum	0	10	0	10
Vitamin content of diet	ABCD	BCD + serum- vitamins	BCD	ACD + serum- vitamins

The salt mixture was based on a formula by McCollum and Davis and suggested by Pryde [1928]. It has the following composition:

	%
NaCl	4.7
MgSO ₄ .7H ₂ O	7.1
NaH ₂ PO ₄ .H ₂ O	9.4
K ₂ HPO ₄	25.8
CaH ₄ (PO ₄).H ₂ O	14.6
Ca lactate	35.2
Fe citrate	3.2
KI	Trace (0.003 %)
	100

The cholesterol used was activated by dissolving 2 g. in 100 cc. warm light petroleum and exposing to rays from a mercury vapour quartz lamp at a distance of 12 ins. for 30 minutes. 40 cc. liquid paraffin were then added, and the ether

Table I.

No. of rat	Diet	Vitamins in diet	Initial weight	Gain in weight	Period of observation (days)	Fate of rat
22	1	A, B, C, D	41	159	56	Survived, healthy
23	1	"	43	142	56	"
24	1	"	43	172	56	"
47	2	B, C, D and serum	29	105	55	"
48	2	"	32	136	55	"
49	2	"	30	141	55	"
57	2	"	38	147	56	"
58	2	"	36	109	56	"
59	2	"	36	179	56	"
60	3	B, C, D	39	39	52	Ataxic
61	3	"	32	28	57	Died
62	3	"	37	51	54	"
50	4	A, C, D and serum	32	—	24	"
51	4	"	32	3	25	"
52	4	"	33	5	25	"

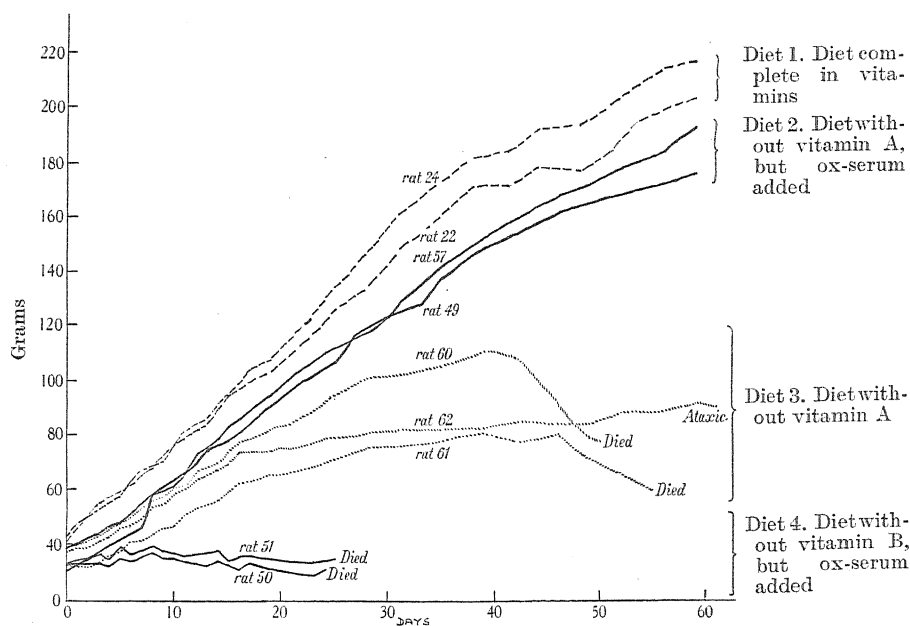


Fig. 1. Chart showing that serum contains vitamin A but not the vitamin B complex.

was evaporated off on a steam-bath. The resulting solution contained 5 mg. irradiated cholesterol in 0.1 cc. oil. This amount was administered daily to the rats by means of a graduated pipette.

In the case of rats receiving cod-liver oil in addition to irradiated cholesterol, a mixture was prepared, 0.1 cc. of which contained 5 mg. of cholesterol and

0.05 cc. cod-liver oil. This was obtained by adding 20 cc. liquid paraffin and 20 cc. cod-liver oil to the irradiated solution of 2 g. cholesterol in 100 cc. light petroleum and evaporating off the ether.

The diets were so prepared that the addition of serum did not increase the calorific value of the diet. Thus when a daily ration of 10 cc. serum was allowed, the corresponding amount of protein was deducted from the caseinogen ration. As the serum used had a protein content of 7.5 % (as determined by the Kjeldahl method), a 10 cc. ration represented 0.75 g. protein: rats receiving serum were therefore given 2.25 g. caseinogen instead of 3 g. daily.

In the latter part of the experiment lemon juice was not added to the diets. This omission, however, was not made until it had been definitely established by other experiments that lack of vitamin C did not affect the rats in weight or in any other way over a period of several months longer than the duration of the present experiment (two months).

15 rats were used in the investigation: 3 were put on diet 1 (complete in vitamins); 6 on diet 2 (containing serum instead of vitamin A); 3 on diet 3 (containing no vitamin A); 3 on diet 4 (containing serum instead of vitamin B). The rats were weighed at least twice a week.

The results are indicated in Table I and Fig. 1.

RESULTS.

Vitamin A. From Table I and the accompanying chart it is clear that serum contains vitamin A, since when serum is added to a diet deficient in vitamin A growth proceeds normally.

Since the average rate of growth of the rats was 2.8 g. per day on diet 1 and 2.5 g. per day on diet 2, the vitamin A content of 10 cc. serum is approximately 90 % of that of 0.05 cc. cod-liver oil; *i.e.* the vitamin potency of the serum used was 1/200 (0.45 %) that of the sample of cod-liver oil used.

Vitamin B. As the animals on diet 4 died in 3-4 weeks with scarcely any gain in weight, it is clear that the vitamin B complex is not present in serum. The possibility exists, however, of one or other of the components of vitamin B being present in serum.

CONCLUSION.

Ox-serum contains vitamin A but does not contain the vitamin B complex.

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CCIV. THE ACTION OF VITAMIN C ON THE OXIDATION OF TISSUES *IN VITRO*.

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(Received August 12th, 1933.)

A FEW years ago, Szent-Györgyi [1928] isolated from the adrenal cortex a strongly reducing substance, "hexuronic acid." He showed that this substance could be reversibly oxidised and reduced, and suggested that it might play the part of an intermediary hydrogen carrier in tissue respiration. Recently, Svirbely and Szent-Györgyi [1932] produced strong evidence that "hexuronic acid"—since renamed ascorbic acid—is identical with vitamin C, and subsequent work has largely confirmed this.

It seemed probable that if vitamin C does play a part in tissue respiration, it might be possible to demonstrate its action by adding ascorbic acid to tissues which had been depleted of their store of vitamin C, that is, to the tissues of animals suffering from scurvy, and testing whether the addition of the vitamin increased the surviving respiration of the tissue. On carrying out the experiment, using the tissues of scorbutic guinea-pigs, it was found that the addition of vitamin C even in very small quantities does bring about a definite increase in the respiration of the tissues *in vitro*.

EXPERIMENTAL.

Guinea-pigs weighing about 300 g. were fed on a scurvy-producing diet consisting of bran, 60 %; rolled oats, 30 %; separated milk powder (heated for 2 hours in the oven at 120° with occasional stirring), 10 %. Water was given *ad lib.* and each animal received 0.5 cc. cod-liver oil weekly. The normal (non-scorbutic) control animals received the above diet with the addition of 3 to 5 cc. of fresh lemon juice daily. On this diet, the animals without lemon juice began to lose weight in about 3 weeks and showed definite symptoms of scurvy, while the control animals continued to grow. The animals were usually taken for experiment after about 3 weeks on the diet, though some were taken earlier than this. Equal numbers of male and female animals were used. The tissues examined were liver and muscle.

The guinea-pig was killed by a blow on the neck and allowed to bleed, and the tissues were quickly taken out and prepared, the liver by cutting thin slices by hand with a razor, and the leg muscle by cutting into small pieces with sharp scissors. Samples of the tissue were then weighed out on the torsion balance and suspended in phosphate buffer at p_{H} 7.4, either with or without vitamin C, in the bottles of the Barcroft differential manometric apparatus. Phosphate buffer was used in the experiments rather than a bicarbonate-Ringer solution, since it was desired to follow the course of the reaction, and in the manometric methods involving the use of bicarbonate buffer in equilibrium with a CO_2 gas mixture, it is only possible to take one final reading of oxygen uptake. The phosphate buffer

was prepared by treating $M/5$ KH_2PO_4 solution with the requisite amount of $N/5$ NaOH and diluting to 4 times its original volume. In the earlier experiments, the liver slices were cut with a razor moistened with buffer solution and were then washed in phosphate buffer, blotted on filter-paper and weighed. It was found, however, that much better agreement between duplicates was obtained if the liver slices were cut with a razor moistened only by the liver tissue fluid itself, the unwashed slices then being well mixed on a watch glass, divided into approximately equal portions with sharp scissors and equal amounts weighed out on the torsion balance. This technique was used in the majority of the experiments described in this paper, and the agreement between duplicate experiments on the same tissue was usually within 5 %.

In parenthesis, it may be mentioned that a very convenient method of checking the constants of the manometric apparatus was found to be given by the oxidation of alkaline ferrous sulphate. A suitable amount (depending on the range of the apparatus) of standard ferrous sulphate solution, slightly acidified with H_2SO_4 , was run into each right-hand bottle from a micro-burette, and, after bringing the apparatus into equilibrium in the bath and closing the taps, excess of NaOH was tipped into the solution. This can readily be done from a small tube suspended on the soda tube of the bottle. The oxygen uptake due to the oxidation of the ferrous iron reaches completion in about 15 minutes, and the method has the advantage that the calibration can be done under the same conditions as those under which the actual experiments are carried out.

The manometers used for the controls without vitamin C and the experiments with vitamin C were frequently interchanged.

Two different samples of ascorbic acid were used in these experiments. Both were colourless, crystalline preparations and, when titrated with iodine, took up exactly 2 atoms of iodine per molecule. The ascorbic acid solution was prepared freshly just before each experiment by dissolving the crystalline acid in a small volume of phosphate buffer and bringing the p_{H} back to 7.4 by adding the theoretical amount of $N/5$ NaOH . An equal amount of ascorbic acid in phosphate buffer but without tissue was always added to the left-hand bottle of the apparatus to compensate for the small oxygen uptake due to the autoxidation of ascorbic acid itself. In all experiments, the total volume of fluid in each bottle was 3.0 cc. The bottles were filled with oxygen rather than with air in order to reduce the possibility that the thickness of the tissue might be a limiting factor in the rate of diffusion and uptake of oxygen. A further reason for using oxygen was that the properties of ascorbic acid suggested that it might function as an intermediary hydrogen carrier between the reducing systems of the cell and activated oxygen. If this were so, its catalytic effect might be expected to be greater if the oxygen-activating systems of the cell were saturated with oxygen.

In filling with oxygen, the technique of Dixon and Tunnicliffe [1923] was used, the vessels being evacuated simultaneously to a pressure of about 100 mm. Hg and allowed to fill with oxygen, the evacuation and filling being carried out three times. The manometers were then shaken in a water-bath at 37° , allowing them to shake in the bath for 10 minutes before closing the taps and commencing to take readings. The CO_2 given off by the tissue was absorbed by rolls of starch-free filter-paper soaked in 40 % KOH in the small inset tubes in the bottles, and the oxygen uptake was measured over a period of 2 hours. About 45 minutes usually elapsed between the killing of the animal and the first reading on the manometer. Most of the determinations were carried out in duplicate on the same tissue both with and without vitamin C, and the mean values of oxygen uptake of the pairs with and without the vitamin respectively were compared.

Table I shows the effect of 0.25 mg. of ascorbic acid (concentration 0.08 mg. per cc.) on the oxygen uptake of liver slices from scorbutic guinea-pigs. The oxygen uptake figures are the totals over a period of 2 hours, and the tissue weights are given as wet weights. All the experiments, with the exception of the first three in the Table, were carried out in duplicate, both with and without addition of the vitamin, a total of 17 scorbutic guinea-pigs being used.

Table I. *Oxygen uptake of scorbutic liver respectively with and without 0.25 mg. vitamin C.*

Tissue weight in mg.	Alone mm. ³ O ₂	+ Vitamin C mm. ³ O ₂	Difference	Percentage increase
154	194	232	38	+20
174	252	272	20	+ 8
237	130	205	75	+57
300	466	541	75	+16
300	527	566	39	+ 8
285	286	370	84	+29
300	400	503	103	+26
225	144	164	20	+14
300	361	547	186	+52
185	145	173	28	+19
100	158	183	25	+16
255	523	600	77	+15
250	357	404	47	+13
152	338	373	35	+10
300	437	458	21	+ 5
207	405	450	45	+11
200	230	349	119	+52

It can be seen that there is a variable but very definite increase in the oxygen uptake of scorbutic guinea-pig liver produced by the addition of 0.08 mg. per cc. of vitamin C.

A few experiments have been carried out with 0.1 mg. ascorbic acid in 3 cc. and a definite increase in the oxygen uptake of scorbutic liver was produced. In one experiment with 0.05 mg. of the vitamin in 3 cc., a completely negative result was obtained.

Table II shows the effect of addition of 0.25 mg. of ascorbic acid in 3 cc. on the oxygen uptake of liver slices from nine normal guinea-pigs. Apart from the first two experiments in the table, the experiments were all carried out in duplicate and the conditions were identical with those in the experiments with scorbutic liver.

Table II. *Oxygen uptake of normal liver with and without 0.25 mg. vitamin C.*

Tissue weight in mg.	Alone mm. ³ O ₂	+ Vitamin C mm. ³ O ₂	Difference	Percentage difference
300	793	762	- 31	- 4
215	625	619	- 6	- 1
*197	91	95	+ 4	+ 4
214	416	367	- 49	-12
200	467	466	- 1	0
232	400	384	-16	- 4
200	328	328	0	0
300	720	659	-61	- 8
283	464	459	- 5	- 1

* In this experiment, the tissue had stood in air for 2 hours before testing.

It is clear that no increase is produced by addition of the vitamin to liver slices from normal guinea-pigs, in fact, if anything, there is a slight decrease in the oxidation in presence of ascorbic acid.

It should be mentioned that the results both in this table and those with scorbutic animals in Table I are in no way selected. They are the results of all the experiments I have carried out with the exception of one which was carried out on a guinea-pig obtained from another laboratory and believed to be suffering from scurvy. In this case, no increase in oxygen uptake was obtained on adding 0.25 mg. ascorbic acid to the liver slices, but on *post mortem* examination no signs of scurvy could be found in the animal.

It was not found possible to correlate the amount of increase in oxygen uptake produced on addition of the vitamin with the degree of scurvy from which the animal was suffering. While most of the scorbutic guinea-pigs showed definite haemorrhages around the knee joint, some of the biggest increases in oxygen uptake were obtained with animals which had been fed for a relatively short time on the scorbutic diet and which, though showing symptoms of scurvy on *post mortem* examination, appeared lively and well nourished. It appears very unlikely, therefore, that the effects observed were due to the inanition which occurs in the later stages of scurvy.

It was observed that in most of the experiments with scorbutic tissue, the accelerating effect of vitamin C on the oxygen uptake became much greater during the second hour than it was during the first hour, and the percentage increase in uptake produced by the vitamin calculated on the second hour alone gave much larger figures than those shown in Table I. Possibly this may be due to the slow diffusion of the vitamin into the tissue, or to a small residual quantity of vitamin C in the scorbutic tissues which becomes used up, the effect of added vitamin C then becoming greater.

No attempt has been made to calculate the results in terms of oxygen uptake per g. of tissue, since it was found consistently that under the conditions of these experiments, the oxygen uptake per g. of tissue is dependent on the amount of tissue taken. Using two different weights of the same tissue, the uptake per g. was always greater with the greater weight of tissue. Possibly some oxidative catalyst diffuses out of the cell into the buffer, and the velocity of oxygen uptake of the tissue depends on the concentration of this substance in the surrounding fluid. It is consequently difficult from the results in Tables I and II to calculate average figures for the oxygen uptake per g. of scorbutic and normal tissues. A further difficulty is that the time elapsing between the death of the animal and the first measurement varied in different experiments. On the whole, however, in comparing experiments in which equal or approximately equal weights of liver were used, it appears that the oxygen uptake of the scorbutic tissue is definitely lower than that of the normal tissue. The addition of ascorbic acid tends to raise the uptake of the scorbutic tissue towards that of normal tissue rather than to increase the uptake above the normal value.

A few experiments have been carried out on muscle, but these experiments have been less satisfactory, since I have been unable to prepare thin slices of muscle, and when chopped muscle is used a great part of the oxygen uptake occurs while the apparatus is being brought into equilibrium in the thermostat. The experiments indicate, however, that both with 0.1 mg. and 0.25 mg. of ascorbic acid, there is a slight increase in the oxidation of the muscle of normal animals and a relatively large increase in the oxidation of muscle taken from scorbutic animals, though too few experiments have been done to justify a quantitative comparison.

Recent experiments by Harris and Ray [1933] indicate that the guinea-pig requires about one mg. a day of vitamin C for normal maintenance. It seems therefore that in the experiments that I have described, in which the effects in oxidation have been produced *in vitro* by the vitamin in concentrations of 0.08 mg. per cc. or less, the vitamin is acting in concentrations which are not unphysiologically large.

That these findings have some relation to events in the intact animal is indicated by a recent paper by Söderström and Törnblom [1933], in which it is shown that feeding guinea-pigs on a scurvy-producing diet results in a marked decrease in the uptake of oxygen by the intact animals.

It is probably premature to attempt to speculate on the relation of these findings to scurvy as a disease. It is not difficult to imagine, however, that impeded oxidation in the scorbutic animal might lead to loss of tone in the capillary muscles, leading to increased permeability and the characteristic haemorrhages associated with scurvy.

Tissues other than liver and muscle have not so far been examined. Gavrilescu *et al.* [1932] have shown that the action of vitamin B₁ concentrates in restoring the lowered oxygen uptake of polyneuritic pigeon's brain is specifically concerned with the glucose-lactate enzyme systems. Experiments designed to determine what oxidising system or systems are linked up with the activity of vitamin C are now in progress.

SUMMARY.

The surviving respiration of tissues taken from normal and scorbutic guinea-pigs was measured manometrically in oxygen, both with and without addition of vitamin C (ascorbic acid). With scorbutic animals, using thin slices of liver suspended in phosphate buffer (p_H 7.4), the oxygen uptake is lower than that of liver from normal animals.

The addition of 0.25 mg. of ascorbic acid in 3 cc. brings about an increase in the oxygen uptake of scorbutic liver slices amounting to 5 to 57 % (average 25 %). With normal guinea-pigs, the oxygen uptake of the liver was unaffected or even slightly decreased by the addition of 0.25 mg. of ascorbic acid. Using chopped muscle, the oxygen uptake with normal guinea-pigs was slightly increased with 0.1 to 0.25 mg. ascorbic acid, while with muscle from guinea-pigs suffering from scurvy, a relatively large increase in oxygen uptake was obtained.

The author is indebted to Prof. E. Mellanby for his interest in this work.

Part of the expense of the work was defrayed by a grant from the Government Grant Committee of the Royal Society.

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CCV. THE EFFECT OF TESTICULAR HORMONE ON NORMAL SEXUALLY MATURE RATS. A METHOD OF BIOLOGICAL ASSAY.

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(Received August 14th, 1933.)

In the previous paper [Korenchevsky *et al.*, 1933, 1] a study was made of the influence of a purified preparation of testicular hormone isolated from human urine on young normal rats, about one month old, *i.e.* on rats sexually immature at the beginning of the injections. The injected animals, as compared with the controls, showed depression of the development of the testes in most cases with small doses and in all rats with large doses. The prostate with seminal vesicles and the penis were, in most cases, slightly decreased in weight with small doses, whilst with large doses an increase in weight was obtained and also an acceleration in the involution of the thymus and a slight increase in the growth of the kidney. No large or constant changes were found in the weights of the other organs or in the body weight. In the present paper a description is given of experiments performed on normal adult rats which had reached sexual maturity at the beginning of the injections.

Technique.

Experiments were performed on 37 rats belonging to 9 litters. The ages and weights of the rats are given in Table I. The age at the beginning of the experiment of the first six litters of 29 rats was from 74 to 77 days. The 4 rats of

Table I. *Age and average weight of the rats in each group of each litter.*

No. of litter	No. of rats in litter	Initial age (days)	Final weight of rats			
			Controls	0.5 R.U.	1 R.U.	10 R.U.
1	6	77	330	313	—	329
2	5	75	305	290	—	312
3	3	75	322	—	325	—
4	5	75	361	—	380	—
5	5	75	312	—	350	297
6	5	74	364	—	—	341
7	4	134	341	—	—	344
8	2	261	476	—	—	401
9	2	About 1 year	595	—	—	456

litter 7 were used in the present experiment about 3 weeks after the end of another experiment in which they had been fed on a diet partially deficient in vitamin D. During the intervening three weeks they were fed on a complete diet with the addition of 5 drops of cod-liver oil daily. For this reason the results with this litter, as well as those with the four much older rats of litters 8 and 9, are not included in the averages in Tables III to VI, but are given separately in these

Tables. The results of litters 8 and 9 have been averaged since each litter consisted of only two rats, one control and one injected rat.

A preparation of testicular hormone, isolated from human urine and dissolved in sesame oil, prepared by Messrs Schering Ltd., was used for the injections¹. This preparation was assayed by us, using our method of assay [Korenchevsky *et al.*, 1932] and was found to contain about 50 rat units per cc. In the experiments two small doses of 0.5 R.U. and 1 R.U. and one large dose of 10 R.U. per day were used, injected in half doses twice a day, the period of injection being 21 days in litters 1 to 7 and 10 days in litters 8 and 9. The rest of the technique was the same as that used by us in our previous experiments [Korenchevsky *et al.*, 1933, 1].

Influence on the weight of the rats.

As can be seen from Tables I and II, neither the difference in the final weight nor in the gain in weight was of such a degree or constancy that any definite significance could be attributed to these with the possible exception of the rats

Table II. *Influence on gain in weight.*

Average gain in weight (g.) in each group of each litter after the injection of testicular hormone.

No. of litter	Actual gain				Gain per 100 g. of initial weight			
	Control rats	Rats injected with			Control	Rats injected with		
		0.5 R.U.	1 R.U.	10 R.U.		0.5 R.U.	1 R.U.	10 R.U.
1	55	42	—	56	20	15	—	21
2	43	44	—	48	16	18	—	18
3	40	—	47	—	14	—	17	—
4	49	—	67	—	16	—	21	—
5	22	—	55	25	8	—	19	9
6	53	—	—	43	17	—	—	14
7	35	—	—	31	11	—	—	10
8 + 9	9	—	—	10	2	—	—	4

injected with 1 R.U. All the rats of this latter group in all three litters gained more than either their controls or the rats of the same litter injected with other doses of the hormone. In litters 3, 4 and 5 the average gain in weight per 100 g. of initial weight of rats was 21.4 %, 31.3 % and 137.5 % more than that of the controls.

Influence on testes.

The results are summarised in Table III. The changes in weight of the testes were small, more particularly when considering the results calculated for the same unit of body weight, being on the average in the three different groups of litters 1 to 6, - 2.7 %, + 5.3 % and - 9.2 % as compared with the average for the control group.

In litters 7 to 9, of older rats, a decrease was noted in the weight of the testes, when calculated per 200 g. of body weight, of 21.1 % and 27.4 %. As can be seen from Table III, a constant although small decrease in the weight of these glands was only obtained with the large dose of 10 R.U., the decrease occurring in all litters except litter 5. A preliminary histological examination of the testes of the injected rats in no case revealed any changes in the seminiferous cells or in spermatogenesis. The Leydig's cells in the testes of rats injected with the large dose of 10 R.U., however, seemed to contain less protoplasm and had smaller nuclei than those of either the control rats or of those injected with 0.5 and 1 R.U. Detailed histological results will be published elsewhere.

¹ We wish to express our thanks to Messrs Schering Ltd., for kindly supplying this preparation.

Table III. *Influence on testes.*

The average weights (g.), actual and calculated per 200 g. of body weight, of testes of normal rats in each group of each litter and their percentage change after the injection of testicular hormone.

		Rats injected with			Percentage change		
No. of litter	Control rats	0.5 R.U.	1 R.U.	10 R.U.	0.5 R.U.	1 R.U.	10 R.U.
A. Actual weights.							
1	2.581	2.408	—	2.173	-6.7	—	-15.8
2	2.952	2.677	—	2.511	-9.3	—	-14.9
3	2.228	—	2.575	—	—	+15.6	—
4	3.215	—	3.211	—	—	-0.1	—
5	2.340	—	2.735	2.292	—	+16.9	-2.1
6	3.076	—	—	2.649	—	—	-13.9
Average	2.732	2.543	2.840	2.406	-8.0	+10.8	-11.7
7	2.022	—	—	1.598	—	—	-21.0
8+9	3.592	—	—	2.111	—	—	-41.2
B. Weights per 200 g. of body weight.							
1	1.565	1.545	—	1.337	-1.3	—	-14.6
2	1.935	1.855	—	1.611	-4.1	—	-16.7
3	1.384	—	1.608	—	—	+16.2	—
4	1.785	—	1.688	—	—	-5.4	—
5	1.500	—	1.577	1.544	—	+5.1	+2.9
6	1.693	—	—	1.554	—	—	-8.2
Average	1.644	1.700	1.624	1.512	-2.7	+5.3	-9.2
7	1.191	—	—	0.940	—	—	-21.1
8+9	1.357	—	—	0.985	—	—	-27.4

Thus with the small doses no definite changes in the testes were obtained, while after the injection of the large dose a slightly depressing effect on the Leydig's cells and in most litters a small decrease, if any, in the weight of the testes were indicated.

Influence on prostate with seminal vesicles and penis.

No definite effect on the weight of these sexual organs (Tables IV and V) was found as the result of the injection of testicular hormone, the variations obtained being small and not constant.

However, it is significant that in most litters a decrease of prostate with seminal vesicles, although slight, was present, indicating that the preparation had no stimulating effect on these organs in adult normal rats.

Influence on thymus.

In all the rats receiving injections of 1 and 10 R.U. the thymus was smaller than in their controls, the average decrease in weight, calculated per 200 g. of body weight, being 23 % and 25.5 % respectively for the six litters of younger rats, while the decrease in litter 7 was 50.4 % and in litters 8 and 9 was 18.6 % (Table V).

Influence on kidneys.

In the previous paper [Korenchevsky *et al.*, 1933, 1] the injection of testicular hormone into young normal rats was shown to be followed by a slight increase in the weight of the kidneys.

In the present experiment this increase was only found consistently in those rats of the first six litters which were injected with 10 R.U. the average increase

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Table IV. *Influence on prostate with seminal vesicles.*

The average weights (g.), actual and calculated per 200 g. of body weight, of the prostate and seminal vesicles of the normal rats in each group of each litter and their percentage change after the injection of testicular hormone.

No. of litter	Control rats	Rats injected with			Percentage change		
		0.5 R.U.	1 R.U.	10 R.U.	0.5 R.U.	1 R.U.	10 R.U.
A. Actual weights.							
1	1.685	1.489	—	1.880	-11.6	—	+11.6
2	1.823	1.298	—	2.088	-28.8	—	+14.5
3	1.482	—	1.476	—	—	-0.4	—
4	2.173	—	2.041	—	—	-6.1	—
5	1.859	—	1.744	1.764	—	-6.2	-5.1
6	2.386	—	—	1.578	—	—	-33.9
Average	1.901	1.394	1.754	1.828	-20.2	-4.2	-3.2
7	2.351	—	—	2.458	—	—	+4.6
8+9	3.596	—	—	2.665	—	—	-25.9
B. Weights per 200 g. of body weight.							
1	1.022	0.954	—	1.151	-6.7	—	+12.6
2	1.195	0.896	—	1.340	-25.0	—	+12.1
3	0.920	—	0.910	—	—	-1.1	—
4	1.205	—	1.072	—	—	-11.0	—
5	1.191	—	0.994	1.188	—	-16.5	-0.3
6	1.314	—	—	0.927	—	—	-29.5
Average	1.141	0.925	0.992	1.152	-15.9	-9.5	-1.3
7	1.379	—	—	1.433	—	—	+3.9
8+9	1.400	—	—	1.252	—	—	-10.6

Table V. *Influence on penis, thymus, kidneys and intra-abdominal fat.*

Average percentage changes in weight (actual and per 200 g. of body weight) of the organs in each group of each litter of the rats injected with testicular hormone, as compared with those of control rats.

No. of litter	Penis			Thymus			Kidneys			Intra-abdominal fat		
	0.5 R.U.	1 R.U.	10 R.U.	0.5 R.U.	1 R.U.	10 R.U.	0.5 R.U.	1 R.U.	10 R.U.	0.5 R.U.	1 R.U.	10 R.U.
A. Actual weights												
1	-14.5	—	-2.5	+18.4	—	-21.0	+0.2	—	+14.4	-5.0	—	+2.2
2	-11.8	—	+9.0	-2.0	—	-11.5	-18.6	—	+6.9	+2.2	—	+1.1
3	—	+4.2	—	—	-23.1	—	—	+17.4	—	—	-27.7	—
4	—	-0.7	—	—	-6.3	—	—	+4.8	—	—	+19.7	—
5	—	-2.1	-1.8	—	-29.3	-48.3	—	+18.5	+14.8	—	+70.7	-19.3
6	—	—	-10.5	—	—	-26.6	—	—	+4.7	—	—	-31.6
Average	-13.2	+0.5	-1.5	+8.2	-19.6	-26.9	-9.2	+13.6	+10.2	-1.4	+20.9	-11.9
7	—	—	+14.6	—	—	-50.2	—	—	+9.4	—	—	+8.4
8+9	—	—	+2.5	—	—	-31.1	—	—	-22.5	—	—	-16.8
B. Weights per 200 g. of body weight.												
1	-9.3	—	-1.6	+24.9	—	-20.1	+5.8	—	+15.1	-1.9	—	+1.7
2	-7.1	—	+6.7	+3.9	—	-13.5	-14.1	—	+4.5	+7.3	—	-1.1
3	—	+3.8	—	—	-23.2	—	—	+16.7	—	—	-28.0	—
4	—	-6.1	—	—	-10.1	—	—	-0.6	—	—	+13.9	—
5	—	-12.0	+3.2	—	-35.6	-45.7	—	+4.8	+20.8	—	+48.9	-15.2
6	—	—	-4.5	—	—	-22.5	—	—	+11.7	—	—	-28.3
Average	-8.2	-4.8	+1.0	+14.4	-23.0	-25.5	-4.2	+7.0	+13.0	+2.7	+11.6	-10.7
7	—	—	+12.5	—	—	-50.4	—	—	+8.7	—	—	+7.9
8+9	—	—	+26.9	—	—	-18.6	—	—	-4.5	—	—	+8.4

being 13 %. The changes in the kidneys of the rats injected with the small doses and in those of the old rats were less constant (Table V).

Influence on other organs.

No definite influence could be seen on the fat deposition (Table V) or on the weights of the adrenals, thyroid, hypophysis, liver, spleen or heart (Table VI).

Table VI. *Influence on adrenals, thyroid, hypophysis, liver, spleen and heart.*

Average percentage changes in weight (actual and per 200 g. of body weight) of organs of the injected rats as compared with those of the control rats.

Organs	No. of litter	Actual weights			Weights per 200 g. of body weight		
		0.5 R.U.	1 R.U.	10 R.U.	0.5 R.U.	1 R.U.	10 R.U.
Adrenals	1 to 6	- 9.1	+ 10.2	- 1.1	- 3.9	+ 4.4	+ 1.8
	7	—	—	- 9.2	—	—	- 11.9
	8 + 9	—	—	- 19.6	—	—	- 3.4
Thyroid	1 to 6	- 12.7	+ 7.1	+ 1.7	- 8.0	+ 0.7	+ 4.2
	7	—	—	- 2.8	—	—	- 4.6
	8 + 9	—	—	- 9.1	—	—	+ 11.0
Hypophysis	1 to 6	- 8.0	+ 7.7	+ 7.0	- 1.7	+ 2.5	+ 9.9
	7	—	—	- 6.7	—	—	- 8.1
	8 + 9	—	—	- 2.8	—	—	+ 22.2
Liver	1 to 6	- 5.9	+ 10.5	+ 3.1	- 0.9	+ 6.2	+ 5.3
	7	—	—	+ 4.1	—	—	+ 3.2
	8 + 9	—	—	- 16.5	—	—	+ 3.8
Spleen	1 to 6	- 6.6	+ 6.9	- 2.2	- 0.9	+ 1.4	+ 0.5
	7	—	—	± 0	—	—	- 2.1
	8 + 9	—	—	- 24.1	—	—	- 3.7
Heart	1 to 6	- 7.4	+ 11.4	+ 0.1	- 2.4	+ 5.5	+ 2.8
	7	—	—	- 3.3	—	—	- 3.1
	8 + 9	—	—	- 13.2	—	—	+ 15.3

DISCUSSION.

The change in weight of the organs, used as a method of assay of different substances as applied to normal rats.

In previous papers [Korenchevsky, 1930, 1932, 1, 2] a method was proposed for the accurate estimation of changes in organs as judged by their change in weight, the weighing being made of the organs fixed in Bouin's fixative. The method gives very accurate results provided the other conditions of the method are fulfilled; its accuracy was proved when applying it to the study of the changes produced by castration and cryptorchidism in male rats [Korenchevsky, 1930] and those produced by testicular hormone preparations in castrated and cryptorchid rats (Korenchevsky *et al.*, 1932; 1933, 1, 2, 3].

The data obtained with adult rats, given in Tables III to VI of the present paper, together with the figures obtained on young rats, given in Tables VIII to XII in the previous paper (Korenchevsky *et al.*, 1933, 1, pp. 570-572], show that the method when applied to normal rats also gives results of a satisfactory accuracy. The variations in the average weights in the different groups of the same litter (where the injection was without effect) were small, especially when the weights of the organs were calculated for the same unit of body weight. These variations become smaller, sometimes even insignificant, if the mean of the average weights of the organs of at least three litters is taken. The importance of this latter consideration has been emphasised in our previous papers.

Taking for example the figure for litters 1 to 6 (Table VI of the present paper) it can be seen that of the 18 figures giving the mean variations of the average changes in weight of organs, as compared with that in control rats of 6 litters, none exceeds 9.9 % and in 13 cases they are less than 5 % when calculated per 200 g. of body weight).

The method may, therefore, be considered to be accurate and reliable also with normal animals and might with advantage be applied, not only to the study of various physiological and pathological conditions, but also to the assay of substances having a specific influence on the weight of one or more organs.

On the influence on normal male rats of the preparation of testicular hormone, isolated from urine.

The strength of the testicular hormone used in the present experiments was similar to that of batch I which was injected into young normal rats in the previous experiments [Korenchevsky *et al.*, 1933, 1, pp. 568-573, see litters 6, 7 and 8]. Therefore, although a special assay of batch I was not made, the doses described in the previous paper as 0.25 D, 0.5 D and 2 D were probably about 1.25 R.U., 2.5 R.U. and 10 R.U. respectively. These doses had a depressing effect on the testes of young rats, more particularly, as is shown by the histological examination, on the seminiferous tissue. As judged by changes in the weights of the organs, the small doses of 1.25 R.U. and 2.5 R.U. had also a depressing effect on the prostate with seminal vesicles and penis, while the large dose of 10 R.U. had a stimulating effect.

In the present experiments on adult sexually mature rats only the large dose of 10 R.U. seemed to have any effect of the kind described above, the weight of the testes being slightly decreased in most litters by this dose. Histologically there was a slightly depressing effect on the Leydig's cells but spermatogenesis and the seminiferous tissue seemed normal.

Therefore, injections started before sexual maturity have a definitely depressing effect on the sexual organs, but if started after sexual maturity the effect, if any, is very slight even with large doses.

Comparison of the data of the present experiments with those of the previous experiments [Korenchevsky *et al.*, 1933, 1] shows that the rate of retrogression of the thymus was accelerated both in sexually immature and mature rats, while the slightly stimulating effect of the testicular hormone on the kidneys was not so marked in the mature as in the immature animals.

No definite changes, in either mature or immature animals, were noticed in the weights of the other organs.

SUMMARY.

1. The changes produced by injections of a purified preparation of testicular hormone extracted from urine were studied on 37 normal adult male rats belonging to 9 litters.
2. The physiological variations from the average of the weights of the organs investigated were small.
3. Therefore, by comparing the mean of the average changes in the litters and following exactly the technique and precautions given in the previous papers, the method described could be applied to normal rats for the study of physiological or pathological conditions or for the assay of substances which influence the weight of one or more organs.
4. The effect of injections of the testicular preparation on adult rats was small, as judged by changes in the weight of the organs and the histological examination of the testes, differing in this respect from the quite definite effect

on some of the organs of young rats, since only the large dose of 10 R.U. seemed to have a slightly depressing effect on the weight of the testes and the Leydig's cells.

5. Acceleration in the rate of retrogression of the thymus was noticed in all the rats injected with 1 and 10 R.U.

6. The increase in the weight of the kidneys was very small, being present in most of the groups injected with the large dose.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due.

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CCVI. SIMULTANEOUS ADMINISTRATION OF TESTICULAR HORMONE WITH ANTUITRIN AND PROLAN OR WITH DESICCATED THYROID.

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(Received August 14th, 1933.)

It is now well known that gonad-stimulating hormones, extracted from the anterior lobe of the hypophysis, or from pregnancy urine, do not influence the secondary sexual organs of castrated animals, since these hormones produce their effects by stimulation of the internal secretion of the testes, or possibly by activation of testicular hormone.

We were unable to find any data in the literature about the action of thyroid hormone on the sexual organs of castrated animals; and as our own investigations on this subject are not yet completed, they will be published later elsewhere. They already show, however, that if there is any influence on the secondary sexual organs of castrated rats it is only slight.

The aim of the present experiments is to investigate whether there is any relationship between the action of testicular hormone, injected into castrated male rats, and that of the thyroid or the anterior lobe of the hypophysis, when introduced simultaneously. By performing these experiments on castrated animals the activity of living testicular cells was excluded. Therefore, any changes obtained differing from the effect of testicular hormone alone should be ascribed to the direct alteration of activity of testicular hormone caused by hormones of the anterior lobe of the hypophysis or of the thyroids.

Since in castrated animals both the latter glands are present, the introduction of an additional amount of the hormones of the anterior lobe or of the thyroid should result in a condition similar to that caused by hypersecretion of these glands.

Technique.

It is unnecessary to discuss here whether the gonad-stimulating substances extracted from urine are completely identical with the hormones of the anterior lobe of the pituitary; in any case their influence on sexual glands is very similar. The preparation from urine was chosen in the present experiments, since such preparations of good quality are much more easily obtained than those extracted from the anterior lobe. We have used "antuitrin S," prepared by Messrs Parke, Davis and Company, for 2 experiments and a mixture of antuitrin S and "prolan," prepared by Messrs Bayer Products Ltd., for 3 experiments (see Table I). These preparations were introduced subcutaneously. Preliminary experiments on young and adult normal male rats have shown that both antuitrin and prolan were very active on the sexual organs in the doses used. As the source of thyroid hormone, desiccated thyroid gland, prepared by Messrs The British Drug Houses Ltd., was given. Each dose of thyroid powder was mixed with about

0.5 g. of wheat germ, moistened with water and given to the rats in small pellets before food; this mixture was greedily consumed in a few minutes. The amount of thyroid fed was calculated according to a special unit of body weight. The daily doses are given in Table I. A testicular hormone preparation, isolated from urine, and prepared and assayed on capons by Messrs Schering Ltd.¹ was used. Different batches of this preparation were used for the various experiments, as it was only supplied in comparatively small quantities at a time. We have not re-assayed the testicular preparations in rat units and in Table I the daily dose is stated in capon units as assayed by Messrs Schering Ltd. Different doses of testicular preparation were given to the rats of different litters (see Tables I and II). The daily doses of testicular and gonad-stimulating hormones were

Table I. *Daily dose of hormones; age and average weight of the castrated rats in each group of each litter.*

No. of litter	Daily dose of hormones				Thyroid in mg. per g. of body weight	No. of rats in litter	Age in days		No. of days after castration at begin- ning of injections	Final weight of rats		
	Testi- cular c.v.	Anti- uitrin S R.U.	Anti- uitrin S + prolan R.U.	At cas- tration			When injections started	Control group		Receiving hormone		
										Testi- cular	Testicular + others	
1	2.5	60	—	—	5	26	48	22	175	206	206	
2	2.5	60	—	—	5	23	45	22	182	202	194	
3	5.0	—	74	—	5	30	52	22	242	219	213	
4	?	—	74	—	4	23	65	42	267	270	270	
5	5.0	—	74	—	5	30	151	121	430	382	386	
6	1.25	—	—	} 1 mg./10g.	8	27	47	20	207	218	207	
7	1.25	—	—		4	23	43	20	—	154	152	
8	2.5	—	—	} 1 mg./ 20 to 100g.	3	24	99	75	242	276	258	
9	2.5	—	—		3	26	94	68	289	267	315	
10	2.5	—	—		4	26	94	68	312	306	291	

Table II. *Average percentage increase for each litter in weight of prostate with seminal vesicles in the group of rats injected with different batches of testicular hormone as compared with that of control uninjected rats.*

No. of batch	Daily dose of c.v. injected	No. of litter	% increase in weight of prostate + sem. vesicles
A. 12	1.25	6	123.0
	1.25	7	No control uninjected rats
	2.5	1	
	2.5	2	
76.4	2.5	8	232.7
	2.5	9	212.0
	2.5	10	211.5
10. 32. 1.	5.0	5	481.0
	5.0	3	173.3
Not marked	?	4	532.0

injected twice a day in half doses, each hormone being injected separately. 24 rats, belonging to 5 litters, were used for the experiments with antuitrin S or prol. and testicular hormone; and 22 rats, belonging to 5 other litters, were used for the experiment with thyroid and testicular hormone. Each litter except No. 7 was divided into 3 groups: Group I, control, untreated rats; Group II, rats injected with testicular hormone only; Group III, rats injected with the same dose of testicular hormone as group II and given in addition antuitrin S

¹ We wish to express our thanks to Messrs Schering Ltd., Messrs Parke Davis and Company and Messrs Bayer Products Ltd. for kindly supplying the preparations.

or antuitrin S and prolan, or thyroid (see Table I). Litter 7, consisting of 4 rats, was divided into two groups only, namely, II and III, without a group of control, untreated rats.

The injections of testicular hormone, antuitrin S and prolan, were all given for 10 consecutive days, but the thyroid-fed rats were given thyroid doses not only during these 10 days of injection but also for 7 days (litters Nos. 6 and 7) or 9 days (litters Nos. 8, 9 and 10) previously, making the total period of thyroid feeding 17 to 19 days. This was done to make the effect of thyroid administration more marked. To save space in Tables II and III only the percentage changes are summarised.

Table III. *Average percentage changes for each litter in weight of organs (calculated per 200 g. of body weight) and in final weight of rats in the group of rats receiving besides testicular hormone other hormones, as compared with that of the group of rats receiving testicular hormone alone.*

No. of litter	Retro-peritoneal fat	Prostate and seminal vesicles	Penis	Adrenals	Thyroid	Hypophysis	Thymus	Liver	Kidney	Spleen	Heart	Final weight of rats
Testicular hormone and antuitrin S or antuitrin S with prolan.												
1	-2.9	+9.0	-1.6	-1.4	-6.9	+6.0	+29.4	-0.5	+0.9	+15.6	-5.1	± 0
2	-19.6	+48.3	+13.1	+8.9	+6.7	-3.8	-3.2	-3.9	+12.5	+23.5	+8.5	-4.0
3	-13.5	+40.9	+6.4	+9.0	+10.7	-2.1	-9.2	-18.1	-0.9	+8.0	+5.8	-2.7
4	-3.3	+10.7	+17.7	+3.9	-10.1	-7.4	-16.3	-0.7	-2.0	+20.7	-4.3	± 0
Average	-9.8	+27.2	+8.9	+5.1	+0.1	-1.8	-0.2	-5.8	-2.6	+16.7	+1.2	-1.7
5	+1.8	-24.6	+3.3	-8.5	+6.8	-19.0	-9.9	-7.6	-2.3	-12.1	-11.9	+1.0
Testicular hormone and thyroid powder.												
6	-17.5	-5.6	-0.8	+17.0	-23.5	+13.5	-9.6	+6.8	+27.3	+47.2	+50.6	-5.0
7	-57.0	-7.1	+3.2	+31.8	-28.8	+10.3	+23.7	+17.1	+35.5	+54.5	+44.9	-1.3
Average	-37.3	-6.4	+1.2	+24.4	-26.2	+11.9	+7.1	+12.0	+31.4	+50.9	+47.8	-3.2
8	-22.6	-2.0	-12.0	+8.9	-11.7	+15.9	+34.0	+3.6	+11.2	+3.0	+7.6	-6.5
9	-6.6	+3.8	-2.8	-23.9	-33.7	-26.6	-24.2	+2.5	+4.7	+12.4	+11.9	+18.0
10	+2.8	+5.1	-3.7	-2.1	+15.0	+14.5	+28.0	-1.9	+2.8	-1.4	+7.9	-4.9
Average	-8.8	+2.3	-6.2	-5.7	-10.1	+1.3	+12.6	+1.4	+6.2	+4.7	+9.1	+2.2

Simultaneous administration of testicular hormone with antuitrin S or with a mixture of antuitrin S and prolan.

This experiment was performed on litters 1 to 5 (Table I). The percentage increase in weight of the prostate with seminal vesicles in the group of rats injected with testicular hormone alone as compared with that of the control group of uninjected rats is shown in Table II (see litters 1 to 5). Different doses of different batches of this hormone produced an increase in weight of the prostate with seminal vesicles of from 123 to 532 %. As can be seen from Table III, in the rats receiving additional injections of antuitrin S or of antuitrin S and prolan, the effect of the testicular hormone was increased, taking an average of 4 litters of young rats, by 27.2 %, but in one litter of adult rats it was decreased by 24.6 %. The percentage increase reached 48.3 and 40.9 respectively, in litters 2 and 3, in two others it was very small.

Such slight and variable results indicate that in the absence of gonads the gonad-stimulating hormones used in our experiments did not influence the activity of the testicular hormone in a constant and pronounced way. From Table III it is also impossible to find a definite marked effect on the weight of the rats, on fat deposition or on the weight of the other organs investigated.

Simultaneous administration of testicular hormone with thyroid powder.

Litters 6 to 10 were used for this experiment (see Tables I, II and III). Litters 8 to 10 were given 1 mg. thyroid powder per 20 g. of body weight in the preliminary period of thyroid feeding, whilst during the period of simultaneous administration of hormones they were given a much smaller dose of 1 mg. per 100 g. of body weight. Litters 6 and 7 received a comparatively large dose, namely, 1 mg. per 10 g. of body weight during the whole period. We have, therefore, divided these 5 litters into two groups: litters 8-10 and litters 6-7. Only in the latter group receiving large doses were constant definite changes observed; as shown in Table III, these changes were a decrease in fat deposition and in the weight of the thyroids and an increase in the weight of the adrenals, liver, kidneys, spleen and heart. All these changes, however, are typical for rats fed with thyroid powder only; and from the results obtained it is possible only to conclude that testicular hormone injections did not counteract the effects of thyroid administration. The typical effect of testicular hormone on sexual organs was not in any way influenced by thyroid feeding (Table III). Smaller doses of thyroid only produced a much less noticeable thyroid effect and did not alter the effect on the sexual organs.

SUMMARY.

1. The effect of a testicular hormone preparation on the sexual, endocrine and other organs investigated of castrated male rats was not altered to a considerable extent by the additional administration of gonad-stimulating hormones extracted from urine or by feeding with desiccated thyroid powder in the doses given.
2. Testicular hormone preparation did not prevent the appearance of the typical changes produced by thyroid feeding, namely, a decrease in the fat deposition and in weight of thyroids and an increase in the weight of adrenals, liver, kidneys, spleen and heart.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work, and to them our thanks are due.

CCVII. HYDROGENASE.

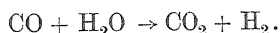
III. THE BACTERIAL FORMATION OF METHANE BY THE REDUCTION OF ONE-CARBON COMPOUNDS BY MOLECULAR HYDROGEN.

By MARJORY STEPHENSON
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(Received August 18th, 1933.)

THE production of methane from a 1-carbon compound was first observed by Sohngen in his classical work on methane formation from fatty acids [Sohngen, 1910], calcium formate being quantitatively decomposed to give methane and calcium carbonate. The same culture also effected the reduction of carbon dioxide. It is important to notice that Sohngen worked with mixed cultures which produced methane also from the higher fatty acids. More recently Fischer *et al.* [1931] have obtained from mud a culture (not claimed by them to be pure) which reduces carbon monoxide to methane, the reaction being accelerated by various colloids. These authors suggest that carbon dioxide is an intermediate product, some evidence being adduced for the occurrence of the reaction

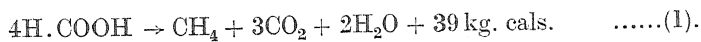


They also showed [1932] that if the ratio of carbon monoxide to hydrogen exceeded one-third, carbon dioxide and methane both resulted, while in higher proportions of hydrogen methane alone was produced.

In the work about to be described we have obtained an organism which we believe to be in pure culture. This differs from the mixed cultures of previous workers in attacking, so far as we have been able to observe, only 1-carbon compounds.

The culture.

The culture used by us was originally obtained from the River Ouse, which had been recently subjected to an influx of fermentable carbohydrate material from a beet-sugar factory and had given a visible fermentation with evolution of gas in the river itself. By subculturing on a medium containing the usual salts [Stephenson, 1930] with 0.5 % formic acid in the form of the sodium salt as the sole source of carbon, and incubating anaerobically, a culture was obtained which decomposed formic acid according to equation (1)



The culture was continued on this medium for upwards of three years, involving some hundreds of subcultivations, and there seems to be no doubt that growth depends on the decomposition of the formic acid as above and is not due to any extraneous source of carbon. After a large number of subcultures on this medium

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the culture appeared morphologically homogeneous, but very numerous attempts to get growth on agar plates failed, and the earlier experiments here described were carried out with no certainty as to whether we were dealing with a pure culture or with an association of organisms of similar physiological properties. Subsequently we resorted to the single cell technique¹ and by this means obtained a culture descended from one cell. The experiments were then repeated with the pedigree culture, the results obtained being identical with those from the crude culture.

As the physiology of the organism is peculiar and not yet fully worked out, we propose to incorporate its full description in a subsequent paper and to confine ourselves here to the chemical aspect of the subject.

Cultivation of the organism.

In our early experiments we found that very large inoculations (1 cc. in 10) were necessary to get certain growth; later we showed that if the old medium were sterilised by passing through a Seitz filter and added to the new medium at the time of sowing a small inoculation served. Finally we found that the Seitz filtrate could be replaced by sulphide (which it always contained owing to the reduction of sulphate). Our final procedure was as follows. The medium to be inoculated was sterilised by autoclaving; immediately before sowing a solution of sodium sulphide in similar medium readjusted to p_H 7 and sterilised by filtration was added, the concentration of the sulphide being arranged to give a final concentration of 0.035 % $Na_2S \cdot 9H_2O$. Immediately after this addition the culture was inoculated and the anaerobic conditions established; delay results in the rapid oxidation of the sulphide. The culture is able to grow indefinitely without the addition of sulphide, provided the sowings are large enough. This is explained by the fact that the bacteria can reduce the sulphate of the medium to sulphide, as is shown in Exp. 7 on p. 1523. Cysteine and reduced glutathione cannot take the place of sulphide in promoting the growth of the culture.

In order to grow the bacteria in bulk six boiling-tubes each containing 30 cc. of medium were first inoculated; when growth was obtained (8 to 14 days) the whole of the contents of the tubes were sown into one litre of medium. A small quantity of sterile mud results in quicker growth at all stages, but is open to the objection that constituents of the mud may be entering into the subsequent reactions. We have tested this point experimentally, and found that the objection is not valid (see Exp. 8, p. 1524); further evidence is given by the fact that identical results are obtained whether the growth of the bacteria has been aided by mud or not. When the culture was well grown, as shown by vigorous effervescence, it was centrifuged and washed with 0.9 % sodium chloride solution or Ringer's solution with full sterile precautions. The growth from one litre of medium was used for each experiment, but it must be realised that the growth is very scanty as compared with that of most organisms grown on broth.

Apparatus.

This consisted of a bolt-headed flask of about 250 cc. capacity attached to a manometer (see Fig. 1). The method used for sterilisation is fully described in a previous communication [Stephenson and Stickland, 1931, 2].

Exp. 1. The decomposition of formic acid into methane and carbon dioxide. This is the reaction occurring during the growth of the organism on the formate

¹ One of us (M. S.) owes Prof. Morton Kahn of Cornell University and his assistant Mrs Schwarzkopf the warmest thanks for instruction and help in the use of this method.

medium; the quantitative nature of the process was established by the use of washed suspensions as follows.

Into the flask were put 10 cc. of phosphate buffer, p_H 6.4, 10 cc. of 0.25 *N* sodium formate, 10 cc. bacterial suspension (from one litre of medium) and 10 cc. of water. The flask was then attached to the manometer as previously described; nitrogen passed over heated copper was then introduced and the process repeated. After filling with nitrogen for the second time the manometer was connected to the flask by turning the tap T_1 and the apparatus placed at 37°. When the manometer reading was constant (after 1½–2 hours) the initial reading was taken, together with the temperature and pressure. When the reaction was complete (4–5 days), *i.e.* when no further change of pressure took place, the final readings of manometer, temperature and barometer were taken, and a sample of gas was removed through tap T_3 for analysis.

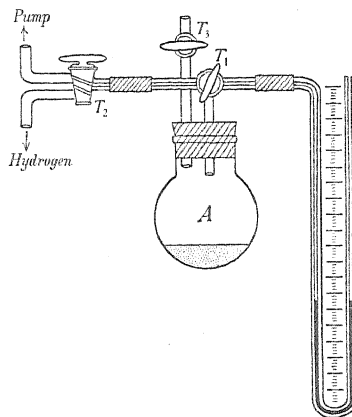


Fig. 1.

The carbon dioxide remaining bound in the solution as bicarbonate was estimated by acidifying an aliquot part and blowing the carbon dioxide into standard baryta by means of a current of air. The complete figures for one experiment are given in Table I and the results of three similar experiments in Table II.

Table I.

Formic acid decomposed (mg.)	115
Volume of gas space of flask (cc.)	168
Final temperature	35°
Final barometric pressure (mm.)	763.0
Final manometer reading (mm.)	+ 108.0
Final composition of gas	H ₂ 0.0 %; CO ₂ 6.35 %; CH ₄ 8.7 %; N ₂ (by difference) 84.95 %
Final CO ₂ in solution	28.2 cc. of 0.0977 <i>N</i> acid
Volumes of gas formed at N.T.P. (cc.)	CO ₂ 41.1; CH ₄ 14.1

Table II.

	Formic acid decomposed (millimols)	CO ₂ formed (millimols)	CH ₄ formed (millimols)	Formic acid 4 (millimols)	CO ₂ formed 3 (millimols)
1	2.00	1.53	0.46	0.50	0.51
2	2.00	1.47	0.45	0.50	0.49
3*	2.50	1.83	0.63	0.625	0.61

* Indicates experiment with pure culture.

Thus a washed suspension of the culture decomposes formic acid quantitatively in accordance with equation (1) (p. 1517). (The last two columns in Table II are added to show the extent of the agreement more clearly.)

Exp. 2. The reduction of carbon dioxide by hydrogen to methane. We next investigated whether the culture responsible for the reaction described in the last section could also effect the reduction of carbon dioxide to methane by molecular hydrogen, as Sohngen's did. The same apparatus was used and in the flask were placed 10 cc. buffer p_H 7.0, 10 cc. bacterial suspension and 20 cc. water. After connection to the manometer, the flask was evacuated; pure carbon dioxide from a Kipp's apparatus was then let in to a pressure of about 60 mm.

(bringing the p_H of the solution to 6.5, the optimum), and this pressure was read off accurately on the manometer attached to the pump; the flask was then completely filled with pure hydrogen, and the tap turned to connect it with its own manometer. Finally, the manometer readings, the barometric pressure and the room temperature were taken; from these data the absolute volumes of carbon dioxide and hydrogen in the flask could be calculated. The whole apparatus was then placed at 37°, and when the uptake of gas had finished (in about 6 days) the temperature and manometer and barometer readings were again taken and a sample of gas withdrawn for analysis. The complete results of a typical experiment are given in Table III, and the condensed results of four experiments in Table IV.

Table III.

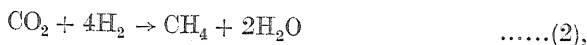
Original pressure of CO ₂ (mm.)	65.5		
Volume of gas space in flask (cc.)	176		
Final composition of gas	CO ₂ 0.0 %; H ₂ 86.7 %; CH ₄ 13.5 %		
	Initial	Final	Difference
Temperature	20°	36°	
Barometric pressure (mm.)	757.5	761.5	
Manometer reading (mm.)	+19.5	-198.5	
Volume of CO ₂ at N.T.P. (cc.)	14.2	0.0	-14.2
Volume of H ₂ at N.T.P. (cc.)	150.0	92.0	-58.0
Volume of CH ₄ at N.T.P. (cc.)	0.0	14.3	+14.3

Table IV.

	Hydrogen used (millimols)	Carbon dioxide used (millimols)	Methane formed (millimols)	Hydrogen used 4 (millimols)
1	2.59	0.64	0.64	0.65
2	2.56	0.63	0.61	0.65
3	2.85	0.70	0.69	0.71
4*	4.48	1.00	0.97	1.12

* Indicates experiment with pure culture.

The theoretical equation is

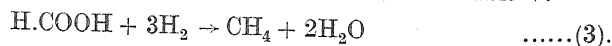


and the results obtained agree with this, so we must conclude that the same culture which decomposes formic acid to methane and carbon dioxide is also capable of reducing carbon dioxide to methane by means of molecular hydrogen.

It will be remembered that Sohngen carried out the fermentation of a number of fatty acids in the presence of hydrogen and obtained greater yields of methane with uptake of hydrogen. We therefore allowed our culture to decompose formate in an atmosphere of hydrogen, and estimated the methane formed and the hydrogen used.

Exp. 3. The reduction of formic acid with hydrogen. The same method was used as in Exp. 1, but only 5 cc. of 0.25 N formate were taken, and the apparatus was filled with hydrogen. After about six days there was no further movement of the manometer, and the usual readings were taken, a sample of gas was withdrawn and the residual formic acid estimated. The results of three experiments are given in Table VI, with complete details of one of them in Table V.

The equation is



From these experiments it is clear that if the change of formic acid into methane is carried out in an atmosphere of hydrogen no carbon dioxide is produced, but the carbon of the formic acid is completely reduced to methane.

Table V.

Formic acid decomposed (mg.)	56.2		
Volume of gas space in flask (cc.)	170		
Final composition of gas	H ₂ 72.6 %; CH ₄ 27.4 %		
	Initial	Final	Difference
Temperature	36°	36°	
Barometric pressure (mm.)	767.0	743.5	
Manometer reading (mm.)	+ 36.0	- 227.5	
Volume of H ₂ at N.T.P. (cc.)	150.0	69.0	- 81.0
Volume of CH ₄ at N.T.P. (cc.)	0.0	26.0	+ 26.0

Table VI.

	Formic acid decomposed (millimols)	Hydrogen used (millimols)	Methane formed (millimols)	Hydrogen used 3 (millimols)
1	1.22	3.62	1.16	1.21
2	1.25	3.91	1.22	1.30
3*	1.11	3.36	1.16	1.12

* Indicates experiment with pure culture.

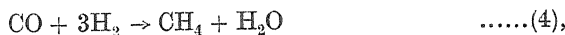
Exp. 4. The reduction of carbon monoxide by hydrogen to methane. The usual phosphate buffer and cell suspension were placed in the flask in a total volume of 40 cc., and the flask was evacuated and filled with a gas mixture containing about 95 % hydrogen and 5 % carbon monoxide, accurately analysed in the Haldane apparatus. The gas was freed from oxygen first by heating a coil of platinum wire in it and then by bubbling it through alkaline pyrogallol on its way to the flask; the former of these two treatments reduced the oxygen content below that detectable in the Haldane apparatus, *viz.* 0.02 %. When the apparatus was incubated at 37°, the gas volume diminished rather slowly, and after several weeks the hydrogen uptake stopped considerably short of the theoretical amount. At the end of the experiment the usual readings were taken, and a sample of gas was withdrawn for estimation of hydrogen, carbon monoxide and methane.

The distinction between carbon monoxide and methane in this mixture depended on the estimation of the oxygen remaining after burning the gas, and as the total amount of (CO + CH₄) was only roughly 6 % of the gas, and the analyses had to be done on samples of 0.8 cc., the results were less accurate than usual. These particular gas samples were always analysed in triplicate, and the values agreed as well as could be expected. Further, a mixture of carbon monoxide and hydrogen, containing no methane, on analysis by this method, gave values for methane between + 0.1 % and - 0.5 %.

Table VII. *Analyses of mixture of H₂ and CO, to test accuracy of analyses of mixtures of H₂, CO and CH₄.*

		(1) %	(2) %			(1) %	(2) %
(a) By ordinary method	H ₂	80.9	80.3	(b) By special method	H ₂	81.3	80.8
	CO	19.1	19.4		CO	19.2	20.0
		<u>100.0</u>	<u>99.7</u>		CH ₄	<u>0.1</u>	<u>-0.5</u>
						100.6	100.3

The results of experiments on the reduction of carbon monoxide are given in Tables VIII and IX. The equation representing the reaction is



and the results are sufficiently close to show that this is actually the reaction that takes place; the experimental error is greater than in the previous experiments.

Table VIII.

Volume of gas space in flask (cc.)	170		
Initial composition of gas	H ₂ 91.6 %; CO 7.3 %; CH ₄ 0.0 %.		
Final composition of gas	H ₂ 90.8 %; CO 3.6 %; CH ₄ 4.1 %.		
	Initial	Final	Difference
Temperature	36°	37°	
Barometric pressure (mm.)	755.0	751.0	
Manometer reading (mm.)	+ 82.0	- 15.0	
Volume of H ₂ at N.T.P. (cc.)	143.5	123.2	- 20.3
Volume of CO at N.T.P. (cc.)	11.4	4.9	- 6.5
Volume of CH ₄ at N.T.P. (cc.)	0.0	5.6	+ 5.6

Table IX.

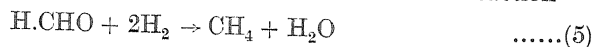
	Hydrogen used (millimols)	CO reduced (millimols)	CH ₄ produced (millimols)	$\frac{\text{H}_2 \text{ used}}{3}$ (millimols)
1	0.91	0.29	0.25	0.30
2*	0.61	0.18	0.175	0.20

* Indicates experiment with pure culture.

Exp. 5. The reduction of formaldehyde by hydrogen. Formaldehyde is far too toxic for use in our apparatus, as the smallest amount the reduction of which could be measured would be sufficient to kill the bacteria and destroy their enzymes. The only possibility was to use some very slightly dissociated compound of formaldehyde, such as hexamethylenetetramine, and this was done.

10 cc. of 0.63 % hexamethylenetetramine were placed in the flask with the usual buffer and bacterial suspension in a total volume of 40 cc., and the apparatus was filled with hydrogen and incubated at 37°. Hydrogen uptake continued for two to three weeks, when the gas was analysed and the remaining hexamethylenetetramine estimated. This was done by converting the hexamethylenetetramine into formaldehyde by treatment with dilute sulphuric acid, allowing the aldehyde to react with sodium bisulphite (in excess) and titrating the bound sulphite with standard iodine in the usual way. The method is unsatisfactory, owing to the fact that the reaction of hexamethylenetetramine with dilute acids produces, besides formaldehyde and ammonia, methylamine, dimethylamine and other products; too low results in the initial and final hexamethylenetetramine estimations would partly account for the low value for "formaldehyde reduced."

The results (Tables X and XI) are sufficient to show that the reaction



does really occur.

Table X.

Formaldehyde disappeared (mg.)	40		
Volume of gas space in flask (cc.)	162		
Final composition of gas	H ₂ 70.1 %; CH ₄ 29.8 %		
	Initial	Final	Difference
Temperature	35°	37°	
Barometric pressure (mm.)	764.0	769.0	
Manometer reading (mm.)	+ 81.0	- 146.0	
Volume of H ₂ at N.T.P. (cc.)	151.4	73.5	- 75.9
Volume of CH ₄ at N.T.P. (cc.)	0.0	32.1	+ 32.1

Table XI.

	Formaldehyde reduced (millimols)	Hydrogen used (millimols)	Methane formed (millimols)	$\frac{\text{H}_2 \text{ used}}{2}$ (millimols)
1	1.34	3.39	1.43	1.69
2*	0.215	0.46	0.20	0.23

* Indicates experiment with pure culture.

Exp. 6. The reduction of methyl alcohol to methane by hydrogen. This was carried out in the usual way except that special precautions were taken to avoid loss of methyl alcohol vapour during the evacuation of the flask. 10 cc. of buffer at p_H 6.3, 7 cc. of water and 20 cc. of bacterial suspension were placed in the flask, which was then twice evacuated and filled with hydrogen, leaving at the end a diminished pressure of about 20 mm. of mercury. 1 cc. of the methyl alcohol solution was then added through the tap, care being taken that no air was admitted, and washed down twice with 1 cc. of water, and the flask was then completely filled with hydrogen and the whole apparatus incubated at 37°. Theoretically, if the reaction taking place is



no change of pressure should occur. The results are given in Tables XII and XIII; in Exps. 1 and 2 excess of methyl alcohol was present, and the amount left was not estimated, but the agreement between hydrogen used and methane formed is good, and in Exp. 3 the methyl alcohol was completely accounted for as methane.

Table XII.

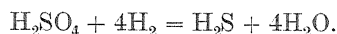
Volume of gas space in flask (cc.)	180		
Final composition of gas	H_2 91.2 %; CH_4 8.6 %		
	Initial	Final	Difference
Temperature	34°	36°	
Barometric pressure (mm.)	753.0	761.0	
Manometer reading (mm.)	+ 74.0	+ 73.0	
Volume of H_2 at N.T.P. (cc.)	165.5	151.0	- 14.5
Volume of CH_4 at N.T.P. (cc.)	0.0	14.2	+ 14.2

Table XIII.

	Methyl alcohol reduced (millimols)	Hydrogen used (millimols)	Methane formed (millimols)
1	—	0.65	0.64
2	—	0.30	0.29
3*	0.62	0.54	0.59

* Indicates experiment with pure culture.

Exp. 7. The reduction of sulphate to sulphide by hydrogen. This experiment was carried out in the same way as those previously described [Stephenson and Stickland, 1931, 2]. The results show that the methane-forming culture can also carry out the reaction



The experimental results are summarised in Table XIV.

Table XIV.

Na_2SO_4 reduced (millimols)	Hydrogen used (millimols)	$\frac{\text{H}_2 \text{ used}}{4}$ (millimols)	Hydrogen sulphide formed (millimols)
0.42	1.60	0.40	0.35

Exp. 8. Control. The bacteria from a litre of medium together with the mud of the medium on which they had grown were placed in the manometer apparatus as usual, but without the addition of any carbon compound. After incubation with hydrogen for fourteen days no methane was found in the gas, showing that the results of the quantitative experiments are not invalidated by the presence of mud. This result received confirmation in experiments in which various 2-carbon compounds were added and in which no methane was formed.

Experiments on reaction velocity.

Of the six reactions described in which carbon compounds are converted into methane, the three involving carbon dioxide and formic acid proceed easily and rapidly and are completed in our manometer apparatus in a few days. The reductions of carbon monoxide, formaldehyde and methyl alcohol, on the other hand, are relatively very slow and are usually incomplete in twenty-one days, even with low initial concentrations. This difference of velocity has been confirmed by experiments in Barcroft apparatus, in which a thick suspension of bacteria (pure culture) was allowed to act on carbon dioxide, formic acid, hexamethylenetetramine and methyl alcohol in an atmosphere of hydrogen. The substrate concentration was in each case $N/30$, and the p_H 6.5; for carbon dioxide the p_H was adjusted by using a mixture of 50 % hydrogen and 50 % carbon dioxide in equilibrium with $N/30$ sodium bicarbonate. The results showed that formic acid and carbon dioxide are reduced at roughly the same rate, while hexamethylenetetramine is reduced at a rate too slow for measurement in the conditions of such experiments. Values for the rate of reaction in terms of the dry weight of bacteria present cannot be given, as the suspension of bacteria is always mixed with a small amount of mud which makes estimations of dry weight or nitrogen content useless.

In these experiments the reaction curve starts immediately in a straight line, which—especially in the case of a very slow-growing organism—precludes the idea that cell multiplication is interfering appreciably with the results.

Experiments on the course of the reactions.

The mechanism involved in the production of methane from these compounds must now be considered. The decomposition of formic acid into hydrogen and carbon dioxide ($H.COOH \rightarrow H_2 + CO_2$) is a common property of many organisms and has recently been shown to be due to a particular enzyme (formic hydrogenlyase) distinct from formic dehydrogenase catalysing the reaction $H.COOH \rightarrow 2H + CO_2$ [Stephenson and Stickland, 1932]. An organism possessing this enzyme together with hydrogenase (activating molecular hydrogen) [Stephenson and Stickland, 1931, 1] and at the same time capable of reducing carbon dioxide might well bring about reaction (1) (p. 1517) in a stepwise manner, by the preliminary decomposition of formic acid into carbon dioxide and hydrogen, followed by a reduction of carbon dioxide by hydrogen as in reaction (2) (p. 1520). Our organism possesses hydrogenase, as is shown by the reduction of methylene blue by hydrogen with a suspension of washed cells:

	Reduction time (min.)
1 cc. 1/5000 methylene blue, p_H 6.3, <i>in vacuo</i>	> 60
1 cc. 1/5000 methylene blue, p_H 6.3, in hydrogen	6

Moreover, we have obtained evidence for the presence of formic hydrogenlyase; a series of test-tubes containing Durham tubes and formic inorganic medium was

sown in the usual way and gas withdrawn from the Durham tubes for analysis at different stages in the growth of the culture. It is seen from the figures in Table XV that in the early stages hydrogen as well as methane was present, but that if the reaction was allowed to continue methane alone was the final product.

Table XV.

Amount of gas (mm.)	Composition of gas	
	CH ₄ %	H ₂ %
2	64	36
6	86	14
7	92	8
8	71	29
21	98.5	1.5
31	99.5	0.5

The extent of growth of the culture was measured by the length of the column of gas in the Durham tube, given in the Table in mm.

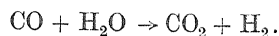
The same conclusion was reached from experiments carried out in Barcroft manometers. A thick cell suspension was shaken in this apparatus with *M/10* formate in buffer at p_{H} 6.5 in an atmosphere of nitrogen; the results showed a brief rapid evolution of gas followed by a slower prolonged evolution. When the gas in the cups was analysed at the end of the first period it was found to contain (besides nitrogen) mainly hydrogen and carbon dioxide with a little methane; after further reaction had taken place the analysis showed decreased hydrogen and carbon dioxide and increased methane.

Analysis of gas in Barcroft cups.

	Total evolution mm. ³	H ₂ %	CO ₂ %	CH ₄ %
After 1 hour	448	0.9	1.1	0.1
After 26 hours	1666	0.6	0.3	3.9

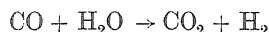
This suggests that formic acid undergoes a preliminary decomposition to carbon dioxide and hydrogen, and methane is then synthesised by reaction (2) (p. 1520). This would involve, not a direct reduction of formic acid, but a reduction by excess hydrogen of the carbon dioxide produced in the first reaction. It must be admitted, however, that we are offering here a plausible hypothesis and no proof.

In respect of reactions (4), (5) and (6) (pp. 1521, 1522 and 1523 respectively) we are in the dark. Possibly a preliminary reduction of all three initial compounds to formic acid and a decomposition of this first to carbon dioxide and hydrogen and thence to methane is as plausible as any. It should be noted that recent workers [Fischer *et al.*, 1932], have suggested that in the case of carbon monoxide a decomposition of water takes place thus

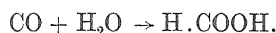


Their evidence rests on the appearance of 13.4 cc. of hydrogen in a bacterial decomposition of 1100 cc. of carbon monoxide in the presence of mud, giving 323 cc. of methane and 724 cc. of carbon dioxide. Without a control experiment

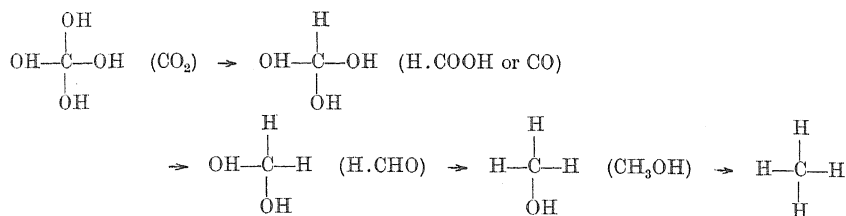
in nitrogen no evidence exists to decide whether carbon monoxide is transformed to methane *via* this reaction



or by hydration to formic acid



As an alternative it is possible that formic acid is a stage in the reduction of carbon dioxide to methane by a stepwise process thus:



On this view formic hydrogenlyase would be competing with the enzyme concerned in the reduction of formic acid, and the hydrogen produced by its action in the initial stages would be finally used up again in the reduction.

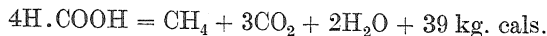
The reduction of other carbon compounds.

We have shown that five simple 1-carbon compounds are reduced by our organism to methane. On the other hand the following compounds were not attacked: acetic, propionic, butyric and caprylic acids (which were all decomposed to methane and carbon dioxide by Sohngen's mixed culture), ethyl alcohol, acetaldehyde and glucose. These compounds were tested in conditions where they formed the sole source of carbon for the organism, and also in broth cultures; in the former case no growth occurred, and in the latter the bacteria grew on the broth but failed to produce any methane from the added compound. In addition acetic acid, acetaldehyde and ethyl alcohol were tested in the manometer apparatus, with washed suspensions, with negative results. As far as we have tested, therefore, only compounds with one carbon atom are reduced by our culture.

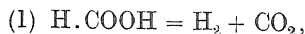
SUMMARY.

1. An organism has been isolated by the single cell technique which is able to live anaerobically on an inorganic medium with formate as sole source of carbon.

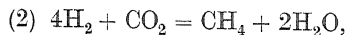
2. The formic acid is decomposed as follows:



This reaction has been shown to occur in two stages, *viz.*



and



and is therefore the work of two enzymes, formic hydrogenlyase (1) and hydrogenase (2) with an additional mechanism for the activation of carbon dioxide as a hydrogen acceptor.

3. The organism (tested in washed suspensions) reduces the following 1-carbon compounds to methane by means of molecular hydrogen (*i.e.* hydro-

genase reactions): carbon dioxide, formic acid, carbon monoxide, formaldehyde (as hexamethylenetetramine) and methyl alcohol.

4. No compounds so far tested containing more than one carbon atom are reduced by this organism with production of methane.

We take this opportunity of thanking Sir Frederick Hopkins for his interest in this work.

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CCVIII. HYDROGENLYASES.

III. FURTHER EXPERIMENTS ON THE FORMATION OF FORMIC HYDROGENLYASE BY *BACT. COLI*.

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(Received August 28th, 1933.)

IN a recent paper [Stephenson and Stickland, 1932] we showed that a culture of *Bact. coli* grown in Roux bottles on tryptic broth was devoid of the enzyme formic hydrogenlyase, while a culture grown in similar circumstances with addition of formate to the medium possessed this enzyme. Yudkin [1932] pointed out that there are two possible explanations of this phenomenon. (1) The difference might be due to a process of "natural selection," or (2) it might be due to a direct chemical action of the formate and broth, stimulating the formation of a new enzyme in the cells. He decided against the former theory, his evidence being (1) the probable uselessness to *Bact. coli* of being able to decompose formic acid anaerobically, and (2) the fact that when a culture (in this case of *Bact. freundii*) grown for many generations on broth containing formic acid was resown on to plain broth, it completely lost its formic hydrogenlyase during sixteen hours' growth (about ten generations). We propose now to offer some further evidence on this point.

For "natural selection" to operate, the formate must have either a beneficial or a harmful effect on the bacteria; in the former case those cells able to profit by the decomposition of formic acid, and in the latter those able to rid themselves of it by decomposition, would have an advantage. In either case, the addition of formate to a young, rapidly growing culture of *Bact. coli* would be expected to have some effect on the subsequent growth rate, and this point was tested experimentally.

Two one-litre flasks containing 500 cc. of tryptic broth were sterilised and brought to 37° by incubation overnight. Each flask was then sown with one drop of a young broth culture of *Bact. coli*, and a sample of each taken for counting. At intervals of 1, 2, 2½, 3, 3½ and 4 hours further samples were withdrawn for counting, to establish the growth rate, and at 4 hours 10 cc. of sterile 25 % formic acid as sodium salt were added to one flask (giving a concentration of 0.5 %) and 10 cc. of sterile water to the other, both solutions having been previously warmed to 37°. After the additions samples were again taken at intervals of 5 minutes, ½, 1, 1½, 2, 2½ and 3½ hours; the counting was carried out by Wilson's viable count method [1922]. The results, shown graphically in Fig. 1, show that the addition of sodium formate had no effect on the growth rate of the culture, so we must conclude that the mode of action of the formate is not "natural selection."

¹ Beit Memorial Research Fellow.

The second alternative, *viz.*, a direct chemical action of the formate and broth on the cells, was therefore provisionally accepted, and we proceeded to try to find further experimental support of this view.

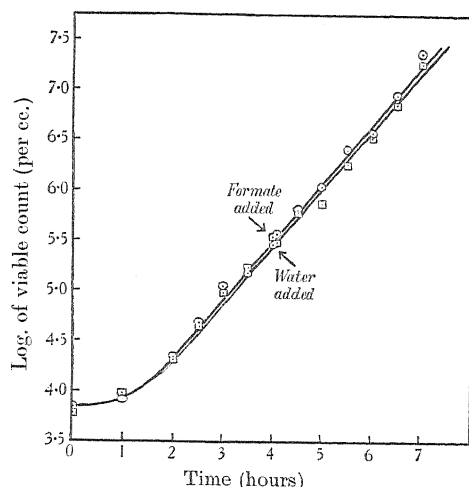


Fig. 1. \square Exp. (formate added); \odot Control (water added).

Growth and enzyme formation.

Our next step was to determine how many generations and how much time were required for the appearance of the enzyme after addition of the formate. This could not be done while growth was still in the logarithmic phase, as at that stage the concentration of bacteria was insufficient for enzyme determinations; the youngest culture from which sufficient bacteria for testing could be obtained by centrifuging was ten hours old, and a culture of this age was consequently used.

A four-litre flask containing three litres of medium, fitted with a rubber stopper carrying a wide glass inlet tube and a siphon, was sterilised and sown with two drops of an eight-hour old culture of *Bact. coli*. Ten hours later, when growth was just visible, a sample of 500 cc. was siphoned off into sterile centrifuge-tubes, and 50 cc. of sterile 12.5 % formic acid (as sodium salt) were added to the remaining culture through the wide tube in the stopper, giving a final concentration of 0.5 % formic acid. Further samples of 500 cc. were run off into sterile centrifuge-tubes at intervals of 1, 2, 3 and 4 hours after the addition of the formate. Each sample as it was obtained was treated as follows. The cups were cooled to roughly 10° by immersion in ice, and a small sample (about 1 cc.) was taken into a sterile dry tube and diluted immediately for viable counting. The remainder was centrifuged and washed once with sterile Ringer's solution. The washed suspensions thus obtained were tested for formic hydrogenlyase (as described by Stephenson and Stickland [1932], and with the help of nitrogen estimations on the bacteria values for Q_{H_2} were calculated. The results of one experiment are given in Table I.

It will be seen that the formic hydrogenlyase had appeared in less than one hour after the addition of the formate, while the viable count had increased by only 18 %, and the Q_{H_2} had reached its maximum after two hours with an increase of only 34 % in the viable count. It appears, therefore, that the enzyme formation

Table I.

Time after addition of formate (hours)	Viable count (10^7 per cc.)	Q_{H_2}
0	5.0	0
1	5.9	450
2	6.7	710
3	8.0	650
4	7.7	650

reaches its maximum during considerably less than one generation time, which fact makes it impossible for the phenomenon to be one of "natural selection."

It might be objected that viable counts in a relatively old culture do not give a true indication of the amount of cell division taking place, as a rapid cell division might be balanced by a high death rate. This objection could be met by using the method of total counting, which would detect any increase of cell numbers with an error of 5 %. This method of counting was used in all subsequent experiments, and at the same time a simpler method was devised for following the course of the production of the enzyme.

The course of the production of formic hydrogenlyase.

A pure suspension of *Bact. coli* containing no formic hydrogenlyase was prepared by growing a culture on plain broth in Roux bottles, centrifuging and washing aseptically. Barcroft cups were sterilised by standing them in chromic acid overnight, washing them six times with sterile distilled water and plugging them with sterile cotton wool plugs from test-tubes; they were dried by leaving them in an incubator for a few hours. The apparatus was set up with 3 cc. of water in the left-hand cup and the following sterile solutions in the right-hand cup:

Tryptic broth (three times normal strength) p_H 7.0	...	1.0 cc.
Sodium formate 1.0 M	...	0.3 cc.
Phosphate buffer 0.2 N , p_H 7.0	...	0.5 cc.
Water	0.2 cc.

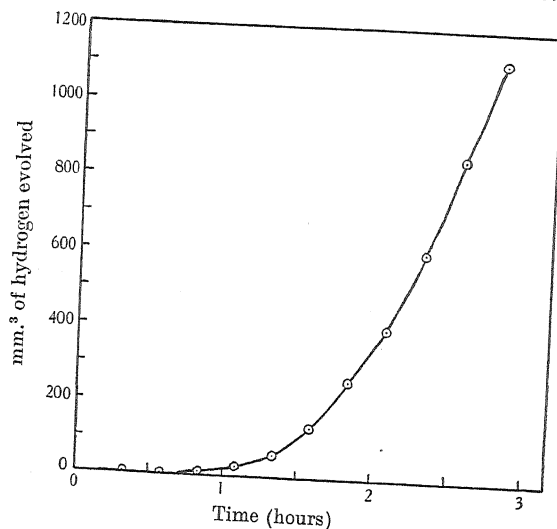


Fig. 2.

1 cc. of a suitably diluted bacterial suspension was then added and the apparatus filled with nitrogen as usual and placed in the bath at 40°.

In Fig. 2 the result of a typical experiment is given, the volume of hydrogen evolved being plotted against time in the usual way, while in Fig. 3 the same experiment is plotted with velocities of hydrogen evolution as ordinates.

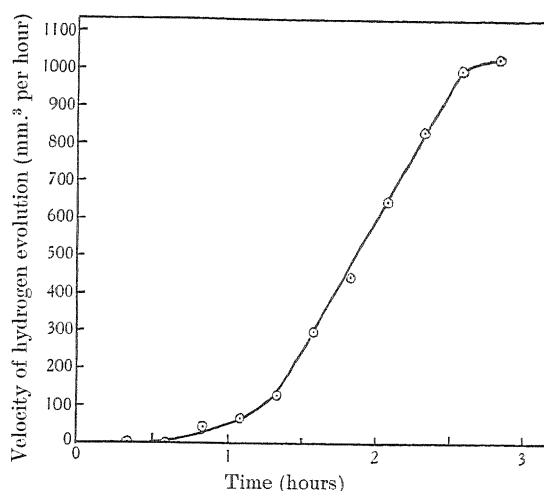


Fig. 3.

Table II.

No.	Duration of exp. (hours)	Total count $10^8/\text{cc.}$		Increase (%)	Q_{H_2}	
		Initial	Final		Initial	Final
1	$2\frac{1}{2}$	1.14	1.19	0	0	3950
2	$2\frac{1}{2}$	5.4	4.9	0	0	3900
3	$3\frac{1}{2}$	1.17	1.12	0	0	3500
4	$1\frac{1}{2}$	2.6	2.6	0	0	2160
5	$3\frac{1}{2}$	1.6	1.5	0	0	1900
6	$5\frac{1}{2}$	1.5	1.5	0	0	1670
7	$4\frac{1}{2}$	2.1	2.1	0	0	1600
8	3	4.3	4.45	0	0	1400
9	3	2.5	2.5	0	0	1400
10	3	5.4	5.2	0	0	1200
11	$2\frac{1}{2}$	2.6	2.5	0	0	1120
12	$3\frac{1}{2}$	7.6	7.9	0	0	870
13	3	7.6	7.6	0	0	770
14	3	9.3	9.2	0	0	760
15	2	1.7	1.7	0	0	750
16	$2\frac{1}{2}$	3.2	3.2	0	0	720
17	3	1.14	1.24	9	0	4850
18	3	3.2	3.6	12	0	900
19	3	1.14	1.39	20	0	3600
20	$4\frac{1}{2}$	2.3	3.15	40	0	1200
21	3	3.2	4.8	50	0	560
22	$3\frac{1}{2}$	1.7	3.0	95	0	1240
23	4	1.7	4.0	120	0	1210

It will be observed that, as would be expected, the initial velocity is zero, and after a latent period the velocity begins to increase. The rate of increase of velocity becomes linear and remains so until the maximum is reached; this may

be interpreted as showing that the synthesis of formic hydrogenlyase is a linear reaction.

In a large number of such experiments a total count was carried out, both on the initial suspension of *Bact. coli* and on the contents of the Barcroft cup after the maximum velocity had been reached; the results are given in Table II. It will be seen that in the majority of the experiments no growth (*i.e.*, less than 5 % increase in total count) took place, while the value of Q_{H_2} increased from 0 to 720–3950, and in the remaining cases (7 out of 23) slight growth (never more than 120 % increase) was found with a similar increase in Q_{H_2} .

DISCUSSION.

The experiments on the growth rate of *Bact. coli* after addition of formate to a young culture, and on the appearance of formic hydrogenlyase after such an addition, are sufficient to show that "natural selection" plays no part in the formation of the enzyme. From the experiments in the Barcroft apparatus just described further conclusions can be drawn. In presence of formate alone no formic hydrogenlyase is developed, but in presence of formate and tryptic broth, after a latent period, the appearance of formic hydrogenlyase proceeds proportionally to the time, while no cell division is taking place. It seems reasonable to deduce from this that enzyme formation is simply a chemical reaction between the medium and the cells. Further work is being carried out to try to elucidate the nature of the reaction.

SUMMARY.

1. This work aims at deciding whether the enzyme formic hydrogenlyase appears in *Bact. coli* by a process of "natural selection" or in some other way.
2. Addition of sodium formate to a young culture of *Bact. coli* has no effect on the subsequent growth rate.
3. The enzyme can be produced by the bacteria while no cell division is taking place, and this production proceeds linearly.
4. The deductions made are that (1) the operation of "natural selection" is impossible, and (2) the formation of formic hydrogenlyase is probably a chemical reaction between the medium and the cells.

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CCIX. NOTE ON THE INCIDENCE OF DERMATITIS AMONG RATS DEPRIVED OF VITAMIN B₂.

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(Received August 31st, 1933.)

THE most consistent sign of a deficiency of vitamin B₂ in rats is failure of growth, but in addition to this an inflammatory dermatitis, considered by some to be analogous to human pellagra, may develop. This dermatitis, however, is irregular in occurrence; some authors have not observed it in any of their animals, others in only a proportion. Various reasons have been put forward to account for this irregularity. It has been suggested that in order to obtain symptoms constantly absolute deprivation of vitamin B₂ must be ensured [Chick and Roscoe, 1928], or, alternatively, that small amounts of vitamin B₂ must be supplied [Sherman and Sandels, 1931]; also that a seasonal variation in incidence of symptoms occurs [Leader, 1930]. Finally the theory has been advanced that the dermatitis is due to lack of some dietary factor, other than that promoting growth, the distribution of which is not as yet understood [Chick and Roscoe, 1928; Sure and Smith, 1930-31]. Kuhn *et al.* [1933] have in fact, stated that vitamin B₂ can be separated into two factors one of which is a skin factor (vitamin H).

In this paper data are given concerning the incidence of dermatitis among the vitamin B₂-deficient animals observed in this laboratory during the past seven years. In addition, results of experiments are reported, in which attempts were made by variations of the diet to influence the development of dermatitis.

EXPERIMENTAL.

Young rats, shortly after weaning, and weighing 30-40 g., were started on the deficient diet. This consisted of caseinogen 20 parts, rice starch 60, salt mixture² 5 and hardened cottonseed oil 15, mixed with an equal weight of water and steamed for 3-5 hours. Cod-liver oil was given separately each day. Vitamins B₁ and B₄ were given as a daily dose of a concentrate from yeast, prepared according to the method described by Kinnersley *et al.* [1933]. The presence of vitamin B₄ in this concentrate was assumed, as extraction of the charcoal was carried out with acid alcohol, in which process both vitamins B₁ and B₄ are stated to be eluted [Reader, 1929].

The type of dermatitis observed in this laboratory in rats deprived of vitamin B₂ has been described by Chick and Roscoe [1927] and is essentially the same as that originally described by Goldberger and Lillie [1926], with the exception that, whereas the lesions observed by them were stated to be bilaterally symmetrical, that has not always been the case with those observed here. Some authors report that the only lesions they obtain as a result of deprivation of

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² Salt Mixture No. 185. (McCollum *et al.*, 1917.)

vitamin B₂ are a scurfy condition with loss of hair. The latter condition is frequently observed among our vitamin B₂-deficient animals but for the purpose of the present study has not been counted as dermatitis.

In working out the proportion of rats developing dermatitis, only those that survived for more than 10 weeks without symptoms have been counted as negatives.

(1) *Number of rats developing dermatitis.* During the past seven years 108 out of the 191 rats on the vitamin B₂-deficient diets have developed dermatitis, an incidence of 57 %. The figures for each of the seven years were: 14/21, 16/26, 23/30, 6/20, 9/21, 16/24 and 24/49.

(2) *Time taken for dermatitis to develop.* The average time during which the rats received the deficient diet before symptoms developed was 10 weeks; in 32 % of the 108 cases the dermatitis occurred after 9–11 weeks of depletion.

(3) *Seasonal variation in the occurrence of symptoms.* The development of dermatitis in the above 108 rats has shown no significant relation to the time of year. Thus 55 % of the rats born in the quarter January–March developed dermatitis, 45 % of those born in April–June, 61 % of those born in July–September and 62 % of those born in October–December. Nor was the period of development of symptoms longer at any one time of year; during the same quarters the average time was 10, 9, 11 and 10 weeks respectively. The experiments were evenly distributed over these periods.

(4) *Weight increase of vitamin B₂-deficient rats.* Considerable variations in weight increase occurred among the animals receiving the same vitamin B₂-deficient diet. The degree of growth, however, did not appear to bear any relation to the occurrence of dermatitis. Thus the average growth during the first five weeks on the deficient diet was 17 g. for the animals which developed dermatitis, 16 g. for those which did not. Nor was a greater weight increase associated with delay in occurrence of symptoms. Rats developing dermatitis after 0–5, 5–10 or 10–15 weeks of depletion had increased in weight by an average of 23, 18 and 15 g. respectively in the first 5 weeks of the observation. This would seem to indicate that those which grew best were most liable to succumb, but the variations in growth were so great (– 3 g. to + 37 g. in 5 weeks) that the above differences in averages were not significant. After the animals had developed dermatitis, growth ceased, as was to be expected since they were then definitely sick.

(5) *The influence of diet.* At first it was thought necessary to use a highly purified caseinogen in the diet in order to induce dermatitis [Chick and Roscoe, 1928]. Later, as the result of papers by Coward, Key and Morgan [1929] and Coward, Key, Morgan and Cambden [1929] showing that unpurified caseinogen did not contain appreciable amounts of either vitamin B₁ or B₂, diets were tested which contained the same (B.D.H. "light white casein") before purification. Substitution of this less pure material for the purified product did not cause any increase in the growth of rats receiving small amounts of either vitamin B₁ or B₂, nor was it found to influence the incidence of neuritis in vitamin B₁-deficient rats or of dermatitis in vitamin B₂-deficient rats.

The results obtained using three different kinds of caseinogen were as follows.

- (i) *Glaxo purified caseinogen (physiological caseinogen, AB).* 13 out of 22 rats (64 %), developed dermatitis after an average depletion period of 8 weeks.
- (ii) *Lister Institute purified caseinogen.* 61 out of 107 rats (57 %), developed dermatitis after an average depletion period of 11 weeks.
- (iii) *"Light white casein" unpurified.* 36 out of 54 rats (67 %), developed dermatitis after an average depletion period of 9 weeks.

There was thus no significant difference between the effects of diets containing these different caseinogens upon the incidence of dermatitis. The increase in weight during the period of vitamin B₂ deprivation was, however, greater when the diet of the rats contained the unpurified caseinogen, the average being 20 g. in the first 5 weeks as against 8.5 g. on diets containing the "Lister Institute purified" caseinogen. This might be taken as an indication that, when unpurified, this caseinogen contained traces of vitamin B₂.

In this connection it may be noted that various authors have found that dermatitis occurred when the rats received some vitamin B₂ and not when complete deprivation was ensured. Sherman and Sandels [1931] obtained dermatitis in rats receiving very small doses of vitamin B₂, but Leader [1930] and Sure and Smith [1930-31] administered large doses of marmite extract or autoclaved yeast (10 % in diet) and obtained symptoms of dermatitis at the same time as good growth. These results are difficult to reconcile with those obtained here, where dermatitis has never developed in rats receiving doses of vitamin B₂, however small, and autoclaved yeast in as small quantities as 0.2 g. daily has been found to cure the symptoms [Chick and Roscoe, 1927].

In the present experiments, the use of unpurified caseinogen which may have contained small amounts of vitamin B₂ was not found to increase the incidence of dermatitis.

In addition to the above the following diets were tried, but no influence on the incidence of dermatitis was detected.

(a) A diet containing 17 % sucrose, with or without supplements of an alcoholic extract of marmite, containing small amounts of vitamins B₁ and B₂ [Leader, 1930].

(b) A diet containing traces only of fat. Evans and Lepkovsky [1929] found that dermatitis occurred more often in rats on a low fat diet.

(6) *Influence of initial reserve stores of vitamin B₂ possessed by the rat.* In observations of the incidence of dermatitis among members of the same litter, great variation has been found; frequently half of the litter succumbed and half did not. Thus it does not appear that some mothers pass on stores of the vitamin to their young while others fail to do so.

DISCUSSION.

The present attempt to discover the factors influencing the incidence of dermatitis in vitamin B₂-deficient rats must be regarded as entirely unsuccessful. Purification of the protein of the diet, to remove all traces of vitamin B₂, was not found to increase the incidence of symptoms, nor was the presence of small amounts of vitamin B₂ in the diet. No seasonal variation in incidence was observed, and no support was obtained for the theory that the irregularity observed was connected with a hitherto unrecognised and uncontrolled dietary factor, differing from that responsible for growth. Further evidence on this last question is given in the following paper [Roscoe, 1933].

SUMMARY.

1. Dermatitis has occurred in 108 out of the 191 rats fed on vitamin B₂-deficient diets in this laboratory during the past seven years. The average time taken for symptoms to develop was 10 weeks.

2. Time of year, weight increase and various alterations in the diet were without effect on the occurrence of symptoms.

My thanks are due to Dr H. Chick for constant advice and criticism throughout the course of this work.

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CCX. THE VITAMIN B₂ CONTENT OF VARIOUS MATERIALS COMPARED BY THEIR POWER TO PROMOTE GROWTH AND TO CURE DERMATITIS RESPECTIVELY.

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(Received August 31st, 1933.)

It has been suggested that the promotion of growth and the prevention or cure of dermatitis are due not to one but to two separate dietary factors, contained in the component of the vitamin B complex known as vitamin B₂ [Kuhn *et al.*, 1933].

Aykroyd and Roscoe [1929]; Chick and Copping [1930]; Chick, Copping and Roscoe [1930]; Roscoe [1930; 1931] showed, however, that all the natural food-stuffs tested which promoted growth when given as source of vitamin B₂ also cured dermatitis. Halliday [1932], on the other hand, in attempts to assay the curative power for dermatitis of various heated preparations of protein-free milk, in some cases obtained growth without amelioration of symptoms. This, however, was attributed to the severity of the dermatitis, and no evidence was obtained from her work or that of the other authors enumerated above that the growth-promoting and dermatitis-curing effects were due to different factors.

In the present paper results are given of experiments in which an attempt has been made to determine the minimum amounts of various materials which will respectively cure dermatitis and promote a given weight increase. It was thought that such a comparison might show a differing distribution of the two factors, if two such exist.

EXPERIMENTAL.

The rats received the diet and vitamin supplements described in the previous paper [Roscoe, 1933, 1].

For the purpose of the curative tests the severity of the dermatitis was graded into three groups as follows.

(1) *Slight inflammation* of paws or nose. Inflammation of the ears or eyelids of a not severe form occurred often and was not necessarily followed by the development of more severe symptoms; it was not therefore reckoned as dermatitis unless subsequent inflammation of other parts occurred.

(2) *Marked dermatitis* of one or more limbs, nose, ears and eyelids; the skin being red, shiny, denuded of hair and sometimes oedematous.

(3) *Severe dermatitis*, showing symptoms as in (2) only more marked, the animal in this stage being very ill.

Before testing the curative action of the materials the symptoms were allowed to proceed to the second stage of marked dermatitis. Cases of slight

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dermatitis may sometimes cure spontaneously. The following materials were tested:

- (i) Watery yeast extracts.
- (ii) Yeast extracts heated for 5 hours at acid reaction.
- (iii) Yeast extracts heated for 1 hour at alkaline reaction.

The preparation of (i), (ii) and (iii) is described in the following paper [Roscoe, 1933, 2].

(iv) Egg-white filtrate, prepared according to the method described by Chick, Copping and Roscoe [1930].

(v) Meat, minced beef steak, dried at 37° and ground.

The results of the dermatitis-curing and growth-promoting tests are given in Table I. For the latter, the criterion was the daily dose needed to promote

Table I. *Daily doses of various materials needed by young rats when given as sole source of vitamin B₂, in order to: (1) promote 50-60 g. weight increase in 5 weeks; (2) cure the dermatitis developing in the absence of vitamin B₂.*

Material	Daily dose g.*		No. of rats			Smallest dose on which more than ½ of the rats were cured, g.	Ratio of growth-promoting to dermatitis-curing doses (a/b)
	required for 50-60 g. wt. incr. in 5 weeks (a)	given to cure dermatitis	Cured	Slight improvement	No improvement		
Yeast extract							
R. VIII	0.25	0.06 0.125	— 2	1	—	0.125	2.0
R. X	0.25	0.06 0.125	— 3	1	3	0.125	2.0
R. XII	<0.25	0.06 0.125	2 1	—	1	0.06	<4.0
Acid autoclaved yeast extract (120°, 5 hours)							
R. V	0.25	0.06 0.125 0.25	— 3 1	1 —	— 1	0.125	2.0
R. X	0.25	0.06 0.125	2 3	1 —	2 1	0.125	2.0
Alkaline autoclaved yeast extract (120°, 1 hour)							
R. VIII	1.5	0.25 0.5	1 1	—	1	0.5	3.0
R. X	0.5	0.125 0.25 0.5	— 3 1	1 1	1	0.25	2.0
R. XII	1.0	0.125 0.25	— 2	—	1	0.25	4.0
Egg-white filtrate							
R. I	10	1.25 2.5	— 2	—	1	2.5	4.0
Meat							
Steak, dry	0.7	0.1 0.2	— 2	1 —	3 1	0.2	3.5

* The doses of yeast extracts are given as the equivalents in dry yeast, those of the egg-white filtrate as equivalents of fresh egg-white.

50-60 g. weight increase in 5 weeks; for the former the smallest daily dose which resulted in cures of more than half the animals. It will be seen that the amount needed to cure dermatitis varied from one half to one quarter of that required to

support the standard rate of growth. The number of rats used for each material was admittedly small so that a variation of this magnitude was not considered significant.

The results indicated that, within the large experimental error unavoidable in this type of experiment, the dermatitis-curing and growth-promoting factors have a similar relative distribution in the materials tested, yeast extract, egg-white filtrate and meat, and that they are similarly affected by heat in acid and alkaline media, both being much more sensitive in the latter case [see also the following paper, Roscoe, 1933, 2]. It is therefore concluded that the two factors are identical.

SUMMARY.

The daily doses of yeast extract, egg-white filtrate or meat needed to cure the dermatitis associated in young rats with vitamin B₂ deficiency were shown to be proportional to the daily doses needed to promote a given weight increase. This was also true of the yeast extract after it had been heated in acid or alkaline media. Thus no support was found for the theory postulating the existence of separate dietary factors responsible respectively for preventing and curing dermatitis and for promoting growth.

My thanks are due to Dr H. Chick for advice and criticism and to Mr F. T. G. Prunty for help with the animals.

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CCXI. THE HEAT-STABILITY OF VITAMIN B₂.

III. THE RATE OF DESTRUCTION AT VARIOUS REACTIONS OF VITAMIN B₂ CONTAINED IN DIFFERENT MATERIALS.

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(Received August 31st, 1933.)

WHILE little is known of the chemical and physical properties of vitamin B₂, it is generally recognised to be the most heat-stable member of the vitamin B complex. This fact made possible its original differentiation from vitamin B₁ and has been the basis of most subsequent methods of separation from other constituents of the vitamin B complex. For this reason much interest and work has been concentrated on ascertaining the degree of this heat-stability and the conditions governing it.

Williams and Waterman [1929] compared the effect on growth of rats of a given amount of brewer's yeast, given as source of vitamin B₂, before and after autoclaving for 6 hours at 120° at varying p_H , and showed that after this length of time destruction was slight in an acid medium but considerable at a slightly alkaline reaction (p_H 8.0) and practically complete at a strongly alkaline reaction (p_H 12-14).

Papers I and II of this series [Chick and Roscoe, 1930; Chick and Copping, 1930] contained the results of experiments on the heat-stability of vitamin B₂ as contained in dried yeast or in a watery yeast extract. The assays were carried out by means of rat growth tests and were checked by cures of dermatitis. It was shown that after heating at 120° for 4-5 hours in an acid medium (p_H 3.3-3.0) about half of this vitamin was destroyed, but after 4 hours in a slightly alkaline medium (p_H 8.3-7.1) nearly all was destroyed, while in a more alkaline medium (p_H 9.9-8.7) destruction was complete.

Halliday [1932] studied the heat-stability of vitamin B₂ present in protein-free milk, in quantitative experiments comparing the minimum amounts of each material needed to produce a given weight increase. Heating for 1 hour at 100° at p_H 4.3, 7.0 and 10.0 caused 10, 30 and 40 % loss, respectively, of vitamin B₂. Four hours' heating at the same reactions resulted in 30, 50 and 75 % loss respectively. It was therefore apparent that the rate of destruction by heat increased with increasing alkalinity and that after 4 hours at a temperature of 100° in a strongly alkaline medium there was great destruction of vitamin B₂.

In apparent contradiction of these results are those of Hassan and Drummond [1927], Reader [1929; 1930], Guha and Drummond [1929], Narayanan and Drummond [1930], Guha [1931, 1], who found that rats grew normally when receiving vitamin B₂ as small daily doses of marmite which had been heated at 110-125° for 1-3 hours in alkaline media, and who therefore concluded that vitamin B₂ was stable to heat and alkali. Only in two cases, however, was the marmite tested for its vitamin B₂ content before heating [Hassan and Drummond, 1927; Reader, 1929] and in no case was a determination made of the minimum doses of the material needed for normal growth before and after heating, so that the only conclusion to be drawn from this work is that vitamin B₂ was not entirely destroyed, in the materials tested, by 1-3 hours' heating in the alkaline medium employed.

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The apparently divergent results obtained by different workers when investigating the heat-stability of vitamin B₂ as present in different materials have led to the supposition that variations in the source of the vitamin might affect the heat-stability. This factor was stressed by Guha [1931, 2]. He investigated the effect of heat (3 hours at 124–125°) on three different materials, (1) fresh ox-liver, (2) a watery solution of commercial liver extract (Eli Lilly, No. 343) and (3) a watery extract of brewer's yeast. In acid medium, no destruction of vitamin B₂ was observed in (2) or (3). But after heating in alkaline medium (initial p_H 9.0, final 7.6–6.1) the vitamin B₂ in (1) and (3) suffered 75 % and 90 % destruction respectively, while that in (2) appeared to be unaffected. These results together with previous ones were taken to show that the vitamin B₂ present in commercial liver extract (and in marmite, see Hassan and Drummond [1927]) was not readily destroyed by heat and alkali as was the case when present in fresh liver or in a watery yeast extract, the greater stability in liver extract and marmite being probably due to the presence of some protective agent.

In this paper therefore, experiments are reported, using various sources of vitamin B₂ similar to the above, *viz.*: a watery yeast extract, a watery liver extract and marmite. These have been heated at 120° for 1 hour in alkaline solution at approximately equal p_H values. In addition, further results are given of the effect of heating yeast extracts at acid and various alkaline reactions.

EXPERIMENTAL.

The method of testing for vitamin B₂ was that described by Chick and Roscoe [1928]. The basal diet and vitamin B₁ and B₄ supplement employed are described in an accompanying paper [Roscoe, 1933, 1]. The young rats weighing 35–45 g. were fed the basal diet only for 1 week during which time their weight was stationary and then received the vitamin B₁ and B₄ supplement and the doses of vitamin B₂. Growth was observed for 5 weeks, the amounts of each material providing sufficient vitamin B₂ to promote 50–60 g. increase in weight in this time being ascertained. This amount of growth was subnormal, and with more vitamin B₂ much better growth occurred; there was therefore no uncertainty, such as occurs when normal growth is the criterion, as to whether the doses administered were indeed the minimum amounts required to support this degree of growth. Four rats received each dose.

The following three materials were investigated.

(1) A watery extract of yeast, made from washed brewer's yeast with boiling 0.01 % acetic acid. 2 cc. of the extract were equivalent to 1 g. of the original yeast (dry wt.).

(2) Marmite, an autolysed yeast extract. This was dissolved in an equal weight of water before heating.

(3) Liver extract (Eli Lilly, No. 343), kindly supplied by the maker in the watery form prepared for intramuscular injection. 1 cc. of the extract was equivalent to 5 g. of the original whole liver.

These materials were all heated for 1 hour at 120° at approximately equal degrees of alkalinity (p_H 8.7–7.2). In addition, tests were made of the yeast extract (1) after treatment for 1 hour at three different degrees of alkalinity and for 5 hours in an acid medium. The heating took place in an autoclave at 15 lbs. pressure. Determinations of the p_H of the materials before and after treatment were carried out by means of a hydrogen electrode.

RESULTS.

The figures in Table I show that when watery yeast extract, marmite and liver extract were heated at 120° for 1 hour at p_H 8.7-7.2, 50 % destruction of the vitamin B_2 took place in each case. Thus there was no significant difference in the degrees of destruction by heat under these conditions of the vitamin B_2 contained in the three different materials.

Table I. *Destruction by heat of vitamin B_2 as contained in different materials.*

The following data are shown.

- (a) The doses in g. of each material found necessary to promote standard growth (50-60 g. weight increase in 5 weeks), or, where this was not found, the nearest dose tested.
 (b) The average weight increase in g. of the four rats receiving (a).
 (c) The percentage destruction of vitamin B_2 , calculated from (a).

Material	Untreated		After heating 1 hour at 120°			p_H during heating
	(a)	(b)	(a)	(b)	(c)	
Yeast extract, R. X	0.25	(69)	0.5	(57)	50 % +	8.7-7.2
Marmite	0.15	(54)	0.3	(51)	50 %	8.6-8.5
Liver extract, Eli Lilly, No. 343	0.075	(49)	0.15	(48)	50 %	8.7-7.7

In Table II are shown the results of a more extensive set of experiments on the heat-stability of the vitamin B_2 in the yeast extract. The results of some previous work with similar extracts [Chick and Roscoe, 1930] are included for comparison.

Table II. *The effect of variation in the p_H of the substrate, and in time of heating, on the destruction of vitamin B_2 contained in a watery yeast extract.*

The following data are shown.

- (a) The doses in g. of each material found necessary to promote standard growth (50-60 g. weight increase in 5 weeks), or, where this was not found, the nearest dose tested.
 (b) The average weight increase in g. of the four rats receiving (a).
 (c) The percentage destruction of vitamin B_2 , calculated from (a).

Yeast extract no.	Time during which material was heated at 120°								p_H during heating
	Untreated		1 hour			4-5 hours			
	(a)	(b)	(a)	(b)	(c)	(a)	(b)	(c)	
R. X	0.25	(69)	—	—	—	0.25	(60)	0 % +	1.4
XII	0.20	(56)	—	—	—	0.4	(60) (5 hours)	50 %	3.3-3.0
XII	0.20	(56)	—	—	—	Trace undestroyed (4 hours)*			8.3-7.1
R. X	0.25	(69)	0.5	(57)	50 % +	—	—	—	8.7-7.2
R. VIII	0.25	(58)	1.5	(53)	83 %	—	—	—	9.0-8.7
R. XII	0.25	(78)	1.0	(60)	75 % +	—	—	—	9.3-8.5
XII	0.20	(56)	—	—	—	Complete destruction (4 hours)*			9.9-8.7

* Results obtained by Chick and Roscoe [1930].

As would be expected, the length of time for which heating was carried on had a considerable effect on the amount of destruction. Thus, while heating at 120° at p_H 8.7-7.2 for 1 hour destroyed only slightly more than 50 % of the vitamin, after 4 hours only traces remained undestroyed; in a more alkaline medium (p_H 9.3-8.5) after 1 hour rather more than 75 % destruction had occurred and after 4 hours (p_H 9.9-8.7) destruction was complete.

The rate of destruction was increased greatly with increasing p_H . While at p_H 1.4 an insignificant amount of the vitamin was destroyed even after 5 hours at 120°, at p_H 3.3–3.0 there was 50 % destruction after 4 hours; at a slightly alkaline reaction, p_H 8.7–7.2, the same percentage destruction occurred after 1 hour's heating, and at p_H 9.0–8.7 there was 83 % destruction after 1 hour.

Some of the above materials were also tested, before and after treatment, for their capacity to cure dermatitis [see Roscoe, 1933, 2]. The results were in good accordance with those obtained by the growth method.

DISCUSSION.

The results here obtained on the heat- and alkali-stability of vitamin B₂ in marmite and liver extracts are at variance with those of Hassan and Drummond [1927] and Guha [1931, 2], who found no appreciable destruction of vitamin B₂ in these materials after 1 or 3 hours' heating in an alkaline medium at 115°, as opposed to considerable destruction in the case of a watery yeast extract. In the present work the vitamin B₂ in marmite and in liver extract was as much affected as that in yeast extract.

The treatment here employed was similar to, but not identical with, that used by the above authors. The marmite was heated in a slightly, as opposed to a strongly alkaline medium [Hassan and Drummond, 1927]; the liver extract was heated at a similar reaction in both instances, but in this work for 1, as opposed to 3, hours [Guha, 1931, 2]. The treatment in the present work was thus in each case less severe, so that less, rather than more, destruction was to have been expected. Hassan and Drummond, however, did not determine the minimum amounts of the untreated marmite needed for normal growth, so that their results, while showing that adequate vitamin B₂ to support growth had survived the treatment, did not prove that some degree of destruction had not occurred. The data in Guha's paper are not given in sufficient detail to show whether this criticism may not be applied to his experiments also.

The results reported in this paper emphasise the fact that the destruction of vitamin B₂ by heat should be regarded as a time-process. As a result of observations on heating for 1 hour [Hassan and Drummond, 1927; Reader, 1929], the vitamin was judged to be very heat-stable in an alkaline medium, whereas Chick and Roscoe [1930], from experiments in which heating was carried out for 5 hours, considered that the vitamin was heat-labile in an alkaline medium. To a certain extent this discrepancy is explained when the different durations of heating are taken into account.

SUMMARY.

1. No difference was found in the stability to heat and weak alkali of vitamin B₂ as contained in (a) a watery yeast extract, (b) marmite solution or (c) a watery liver extract (Eli Lilly, No. 343). When heated for 1 hour at 120° at p_H 8.7–7.2 all three materials lost 50 % of their original vitamin B₂ potency. Thus no support was obtained for the theory that the resistance of vitamin B₂ to heat and alkali varies according to the material in which it is present.

2. In confirmation of previous work vitamin B₂ was found to be relatively heat-stable in acid solution and the rate of destruction to be increased rapidly with increasing alkalinity.

My thanks are due to Dr H. Chick for constant advice and criticism and to Mr F. T. G. Prunty for help with the care of the animals.

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CCXII. THE MICRO-DETERMINATION OF BROMINE IN BLOOD.

By ARTHUR GORDON FRANCIS AND CECIL OWEN HARVEY.

From the Government Laboratory, London.

(Received August 26th, 1933.)

FOR certain biochemical work it is necessary to determine the bromine content of blood in circumstances which preclude the use of samples of greater volume than 1 or 2 cc.

Experience gained during an investigation carried out for the Medical Research Council on methods for the determination of iodine in blood suggested that a method of destroying organic matter involving ignition with alkali under controlled conditions might prove to be satisfactory, and that the bromine might subsequently be determined by a micro-modification of the chromic acid aeration process [Baughman and Skinner, 1919; Evans, 1931].

In the chromic acid aeration processes described by these workers, a double aeration is necessary when chlorides are present, to eliminate errors due to a small amount of chlorine evolved during the first aeration.

In attempting to evolve a micro-method of aeration, a considerable amount of work has been done, the earlier results being promising but erratic at times. When a double aeration was employed, the recovery of bromine varied, and was sometimes as low as 60 %.

As the amounts of bromine to be determined are small (about 0.02 mg.) some variation in the recovery is to be expected, but it appeared that, with a method employing double aeration, the losses would be higher than desirable.

In the later experiments a chromic-phosphoric acid mixture was used for liberating the bromine, and it was found that the conditions could be so adjusted that satisfactory figures were obtained by one aeration in the presence of an amount of sodium chloride approximating to that present in 2 cc. of blood (Table I). The recovery of bromine is within ± 10 % of the theoretical, which is satisfactory for work of this kind.

Table I. *Determination of potassium bromide added to sodium chloride.*

	Bromine added mg.	Bromine found mg.
Control, 20 mg. of bromide-free NaCl	Nil	Nil
Ditto plus 0.005 mg. of bromine (KBr)	0.005	0.0049
" 0.010 "	0.010	0.0099
" 0.020 "	0.020	0.0214
" 0.030 "	0.030	0.0327
" 0.050 "	0.050	0.0515

Preparation of reagents.

Distilled water. The water should be treated with a mixture of potassium carbonate and potassium permanganate and distilled to remove bromine compounds and organic matter.

N potassium hydroxide. Prepared from specially selected material or freed from bromine by electrolysis.

20 % solution of sucrose. This solution is added to ensure that in the "blank" determinations, any bromate present is reduced to bromide. In the actual blood determinations, any bromate will be reduced during the incineration of the blood. The sucrose solution should be substantially free from bromine compounds, but it is added to the blood determinations as well as to the "blanks" so that any correction necessary for bromine present in the sucrose is made automatically.

Chromic-phosphoric acid mixture. (1) Stock solution. Pure syrupy phosphoric acid, sp. gr. 1.75, is treated with sufficient chromic acid to produce a saturated solution and leave an excess of chromic acid undissolved, when the mixture is heated in a boiling water-bath for 3 hours, with occasional stirring. This procedure also destroys organic matter and decomposes any chloride or bromide.

The mixture is allowed to cool overnight, and is then freed from excess of chromic acid by filtration through glass wool. The solution is now again heated in a boiling water-bath and is aerated for 3 hours with a vigorous stream of air to remove chlorine or bromine. The air should be filtered through a plug of cotton wool to remove dust and then through a plug of glass wool to remove cotton wool fibres. After cooling, the solution is ready for dilution with the special distilled water.

(2) Diluted solution. Before being used under the conditions of aeration to be described later, the stock chromic-phosphoric solution must be diluted slightly so that, with 20 mg. of bromide-free sodium chloride, under the standard conditions of aeration, no iodine is liberated in the potassium iodide bubblers, and the recovery of bromine from potassium bromide in the presence of the sodium chloride is satisfactory (see Table I). Dilution of 90 cc. of the stock solution with 5.0 cc. of water usually provides a solution of the required concentration, but it is necessary to examine every batch of stock solution to ascertain whether it functions properly when so diluted.

2 % aqueous solution of potassium dichromate.

2 % solution of potassium iodide. This solution should be prepared from distilled water freed from dissolved gases by recent boiling. When protected from the action of daylight and oxidising vapours, the solution is quite stable, and a fresh solution need only be prepared every 2 or 3 weeks.

0.5 % solution of soluble arrowroot starch. This solution, which must be freshly prepared, is obtained by rubbing up the starch with a little water, and pouring the mixture into boiling water. The total time of boiling should not exceed 2 minutes.

Standard N/500 solution of sodium thiosulphate. This solution is made with distilled water freed from dissolved gases by boiling and containing 1 % by volume of pure amyl alcohol, as recommended by Aitken [1930]. It should be stored in a container fitted with a soda lime tube and a siphon made entirely of glass terminating in a glass tap. When shielded from direct daylight, the solution is quite stable, but should be restandardised at least once a week. The solution may be standardised against a solution of potassium bi-iodate, N/100 in oxidising power.

Destruction of the organic matter.

1-2 cc. of blood, carefully measured or weighed, are placed in a round-bottomed nickel dish, 9 cm. in diameter, and treated with 20 cc. of water, 3.0 cc. of N KOH, and 0.1 cc. of the sucrose solution. The mixture is well stirred with a glass rod, the rod being rinsed with a little water and removed.

After taking the solution to dryness on the steam-bath, the dish and its contents are placed in an oven at 150–160° for 1 hour and then ignited for 1 hour in a muffle furnace at 480–500°.

The material is now moistened with about 20 cc. of water, the carbon is thoroughly broken up with a glass rod, and the mixture is allowed to digest on the steam-bath for 2–3 minutes while being stirred with the rod.

The hot solution is now decanted through a filter (9 cm. Whatman No. 5 paper previously thoroughly washed with hot distilled water before use) into a 100 cc. beaker, the carbon in the dish being again extracted by digestion with water on the steam-bath and finally extracted with water until the filtrate has a volume of about 40 cc. The filtrate is evaporated on the steam-bath in a recently ignited platinum dish, about 8 cm. in diameter.

The filter-paper is placed in the nickel dish, and the contents of the dish are moistened with water and treated with 1.0 cc. of *N* KOH. After being mixed thoroughly, the mixture is evaporated to dryness on the steam-bath, dried for 15 minutes at 150–160° and ignited in the muffle furnace at 480–500° for 1 hour.

The residue, which now contains only a little carbon, is extracted by digestion with water on the steam-bath in the manner already described, the filtrate being added to the previous filtrate which has been evaporating in the platinum dish.

(Note. The nickel dishes should be freed from carbon after use by igniting them in the muffle furnace.)

The combined filtrates in the platinum dish are evaporated to dryness, the dish is placed in the oven at 150–160° for 10–15 minutes and is then ignited for 10 minutes in the muffle at 480–500°.

The residue is dissolved in a little water, the solution is evaporated, and the dish is placed in the oven at 150–160° for 10–15 minutes and ignited for 10 minutes in the furnace at 480–500°.

The moistening, evaporation, drying and ignition for 10 minutes are repeated once again.

To assist the final removal of traces of organic matter, the residue is now dissolved in a little water, treated with 1 cc. of 2 % potassium dichromate solution, and the evaporation, drying and ignition for 10 minutes at 480–500° are repeated.

The alkaline residue is dissolved in about 15 cc. of cold water and the solution is passed through a filter (7 cm. Whatman No. 42, previously well washed with hot water), washing with cold water until the filtrate has a volume of 30–40 cc.

The filtrate is evaporated in the platinum dish; the dish is placed in the oven at 150–160° for 10–15 minutes and is finally ignited for 5 minutes at 480–500°.

The dish is now covered to prevent contamination by organic matter in the form of dust from the atmosphere.

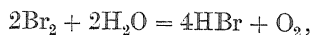
Aeration and titration of the liberated iodine.

The apparatus (Fig. 1) must be kept scrupulously clean and free from minute traces of organic matter. On no account must lubricating grease or rubber joints be used, the ground joints being lubricated with distilled water only.

Frequent cleansing by soaking in chromic-sulphuric cleaning mixture is essential, and this should be followed by very thorough washing, first with tap water, and then with distilled water. Finally, the entire apparatus, with the exception of the two small potassium iodide-starch containers, is rinsed with

very dilute sulphuric acid (approx. $N/5000$), well drained and set up in a position where it is screened from the action of direct sunlight.

(Note. The action of daylight encourages the reaction,



which reaction, should it take place in the tubes D and E , will prevent the bromine from being completely evolved. Similarly, if the tubes are not kept slightly acid, but tend to be alkaline, evolution of the bromine will be incomplete.)

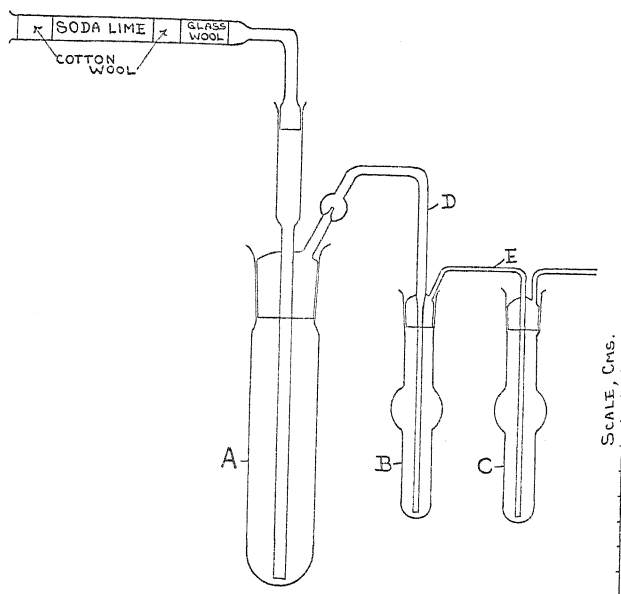


Fig. 1.

Each of the small bubblers B and C contains 0.5 cc. of the potassium iodide solution and 0.5 cc. of the starch solution¹.

The residue in the platinum dish is dissolved in exactly 3.0 cc. of water, and the solution is carefully poured down the funnel into the large bubbler A , the dish being twice rinsed with exactly 1.0 cc. of water, the total volume of water used being 5.0 cc.

The water-pump is now turned on, and a slow stream of air at the rate of about 3 litres per hour² is passed through the apparatus, while 5.0 cc. of the diluted chromic-phosphoric mixture are carefully run down the funnel into the large bubbler. The heat of reaction causes the temperature of the solution to rise slightly.

The soda-lime tube is replaced, and, in about 15 secs. when the effervescence ceases, the rate of the air stream is increased to 8–10 litres per hour and maintained at this rate for 20 minutes (laboratory temperature 15–20°).

The contents of the second small bubbler C , which should contain little or no liberated iodine, are now blown back into B , and the end of the tube D is

¹ The addition of starch to the bubblers is very beneficial in assisting the retention of the liberated iodine [Thomas and Cross, 1928; Zepf and Vetter, 1930].

² If the air stream is too rapid, small drops of the alkaline solution may be splashed into the upper part of the large bubbler and may absorb some of the evolved bromine.

rinsed with a little water, whereby the total volume of liquid in the container of bubbler *B* is brought up to 3-4 cc.

The iodine is now titrated with *N*/500 thiosulphate, using a micro-burette. In the work for the Medical Research Council previously mentioned it was found necessary to make a small correction for the end-point error. In terms of bromine, for small volumes, this correction is +0.0002 *V* mg., where *V* = the volume of the solution after titration (*i.e.* one adds to the figure for bromine obtained by titration, 0.0002 mg. for every cc. of solution).

Some results obtained by the method are given in Table II. These form a complete series of results and are not selected figures.

Table II.

Recovery of bromine compounds added to 2.0 cc. of horse blood in each case.

Results obtained by deducting the figures found for the bromine naturally present in the blood.

Form in which the bromine was added to the blood	mg. of bromine added	mg. of bromine found
Potassium bromide	0.010	0.011
"	0.020	0.022
"	0.030	0.030
<i>o</i> -Bromobenzoic acid	0.010	0.012
"	0.020	0.019
"	0.016	0.017
"	0.024	0.027
"	0.030	0.031
"	0.024	0.027
"	0.030	0.031
"	0.040	0.040
Bromodiethylacetylurea	0.010	0.010
"	0.016	0.015
"	0.026	0.026
"	0.030	0.030

Bromine found in samples of blood kindly supplied by Dr J. B. Orr.

		Total mg. of bromine found	mg. of bromine per 2.0 cc. of blood corrected for "blank"
"Blank"		0.0007	—
"		0.0009	—
*1. Cow	0.98 cc.	0.0171	0.033
"	1.94 "	0.0349	0.035
2. Guinea-pig (F).	0.97 "	0.0217	0.043
"	1.92 "	0.0492	0.050
3. Fowl (M).	0.98 "	0.0075	0.014
"	1.94 "	0.0134	0.013
4. Sheep (F.)	0.94 "	0.0092	0.018
"	1.86 "	0.0171	0.018
5. Rat (M).	0.93 "	0.0116	0.023
"	1.84 "	0.0198	0.021

* This animal had been receiving a diet containing potassium iodide. The figure obtained for bromine may therefore be slightly higher than the true value.

Frequent "blanks" should be carried out to ensure that bromine is not being picked up from the atmosphere of the laboratory or other sources. If the blood is oxalated, then the "blanks" must contain a corresponding amount of the oxalate.

Satisfactory results will only be obtained when strict attention is paid to details. The aerations must be carried out in precisely the manner described, and constant vigilance must be exercised to ensure that errors are not creeping in from unexpected sources. It appears to be desirable to call attention to the following points.

1. The presence of minute traces of organic matter in the apparatus may cause low results as also may the presence of foreign organic matter in the starch solution.

2. If the potassium iodide or starch solutions are alkaline, the results will be low.

3. It is not permissible to use chromic-phosphoric mixture which evolves a small amount of halogen on aeration and to correct for this halogen by means of a "blank." Such a "blank" may be due to elemental chlorine in the chromic-phosphoric solution, which, while being evolved in the "blank," may not be evolved in the actual determination owing to its interaction with the bromide present.

4. High results may be obtained with materials containing iodine. Normally, blood does not contain sufficient iodine to affect the bromine figures.

5. For the recovery of volatile bromine compounds, such as bromoform, the blood would have to be evaporated and burnt in a closed system. The figures obtained (Table II) indicate that the method of alkaline ignition in open dishes is satisfactory with bromine compounds of the nature of those likely to be present in blood.

6. If colloidal silica were present in the large bubbler during aeration, the removal of the bromine would probably be incomplete. Such silica might be derived from the caustic potash or from the material undergoing examination, but no trouble of this kind has been experienced while working with the samples of blood.

7. Examination of the results embodied in Tables I and II shows that, although no chlorine is evolved when working with pure sodium chloride, the figures for bromine tend to be high rather than low, although it was found that, in the absence of chloride, the chromic-phosphoric aeration process always gave low results for the recovery of bromine (about 85 % recovery). As this error tends to increase with increased bromine concentration it appears that, when carrying out a series of comparative determinations of blood-bromine, the results will be more strictly comparable if the volumes of blood taken for analysis are such that approximately the same quantity of bromine is recovered in each case.

SUMMARY.

A method is described for the determination of bromine in small volumes of blood with an accuracy of approximately 10 %.

In conclusion, we wish to thank Sir Robert Robertson for granting us permission to publish this paper, and Dr J. B. Orr for arranging for the supply of samples of blood.

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CCXIII. THE PRODUCTION OF MUCUS DURING THE DECOMPOSITION OF PLANT MATERIALS¹.

I. THE EFFECT OF ENVIRONMENTAL CONDITIONS.

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(Received July 27th, 1933.)

It has been observed that the straw in some manure heaps undergoing decomposition develops stickiness. Such sticky manures may be better adapted to light sandy soils and are possibly unsuitable for heavy clays. The possible effect of mucilage from plant residues on the physical behaviour of the soil has been suggested by Hutchinson and Clayton [1919]. The substance conferring on manures the physical property of stickiness will be referred to hereafter as mucus. It is apparent that mucus formation must be a result of the microbiological activity during decomposition.

Though the decomposition of plant materials has been studied extensively and the losses of individual constituents followed in detail by Rege [1927], Waksman [1928] and Norman [1929], no reference is made in the literature to the production of mucus.

The experiments described in this paper were designed to examine the conditions involved in the production of mucus. Straw was fermented in the presence of different sources of available nitrogen with changes in the physical conditions and reaction. Extractions of the decomposed straws were made to seek any possible correlation between the rates of their decomposition and the production of mucus. The amount of mucus produced was measured by a specially devised physical test.

Physical test for measuring the stickiness.

The principle of the method consists in measuring the vertical force required to separate after drying two metal plates which contain between them a known weight of the manure sample.

The following apparatus is required for the determination—a number of pairs of metal plates, one of 4 ins. square and the other of 3 ins. square with a hook in the centre, a system of two frictionless pulleys in a horizontal plane, lead shot, a receptacle to receive the lead shot and a lead weight weighing 1600 g. with a slit to fit over the hook of this plate. This apparatus is represented diagrammatically in Fig. 1.

The metal plates should be sufficiently rigid not to bend under the forces applied. In order to keep the lower plate firmly fixed, while the force is being

¹ This paper is an abridged form of a part of the thesis approved for the Degree of Doctor of Philosophy in the University of London.

applied, it is made to slide in between two jaws made by screwing to the bench top two metal pieces over another of 4 ins. length and of the same thickness as that of the plates. The lower two plates are kept parallel and 4 ins. apart in such a way that the lower metal plates of 4 ins. square can be easily slid in with no danger of causing any jerks to the plates containing the manure. When the plates are ready for measuring the pull, the lower plate is gently slid in, the loop at one end of the string over the pulleys is put in the hook of the top plate and

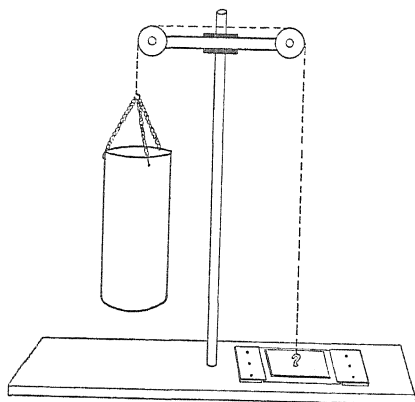


Fig. 1. Apparatus used for measuring stickiness.

the other is connected with the receptacle for carrying the lead shots. The shots are gradually poured in till the top plate is vertically lifted instantaneously from its position of rest. The weight of the shots *minus* the weight of the top plate gives the total pull to separate the two plates. The diameter of the circular block occupied by the manure is measured. An average of three or four readings in different positions is taken as the block is not usually regular. Knowing the diameter and the pull recorded, the force per unit area can be calculated.

The procedure consists in cutting the rotted straw as finely as possible, after which the mass is thoroughly mixed and vigorously worked with a spatula to obtain an even mixture. One gram portions are then weighed out on each of the plates. The mass is gathered into a mound by the spatula in the centre of the plate and the top plate placed in such a way that it rests freely with faces parallel. It is then pressed with the lead weight for 15 seconds and dried at 100° in an oven overnight to ensure uniform and complete drying. It is essential in all the experiments to keep constant the weight and time for pressing the plates, because the area of the manure and its height or thickness depend upon the force with which the top plate is pressed against the manure surface, whilst the pull required to separate the two plates depends upon the distance between them and therefore upon the thickness of the manure disc. In his early experiments on the stickiness of soils Kachinski [1930] left a weight on the sample for a specified time. Bouyoucos [1932] however pressed the plates with the hands, introducing different pressures at different times, without considering the thickness of the disc occupied by the soil.

Table I gives the significance of the determinations of stickiness made by the physical test. It will be seen that the magnitude of the error increases inversely with stickiness and that with highly sticky and moderately sticky samples the figures for stickiness are significant.

Table I. *Significance of the determinations of stickiness made by the physical test.*

Readings	Actual pull recorded in g.	Radius of the manure disc in cm.	Force per unit area per 1 g. of wet sample	Force per unit area per 1 g. of dry sample
NaNO ₃ rot: dry matter 13 %. Very sticky.				
1	5200	1.25	1059	8140
2	4875	1.25	991	7620
3	4958	1.25	1010	7770
4	4800	1.20	1061	8160
5	4110	1.25	837	6436
6	5190	1.25	1056	8120
7	5000	1.25	1018	7830
	5075	1.25	1034	7944
Mean				7752.5
Standard deviation				566.32
Standard error of the mean				± 200.2
Urea rot: dry matter 20 %. Moderately sticky.				
1	2000	1.30	481.4	2407
2	1800	1.25	366.4	1832
3	1200	1.25	244.5	1222
4	1600	1.20	353.7	1768
5	1900	1.20	420.1	2100
6	2000	1.25	407.3	2036
7	2050	1.25	417.4	2087
8	1800	1.20	390.0	1990
Mean				1930.2
Standard deviation				345.0
Standard error of the mean				± 121.9
(NH ₄) ₂ CO ₃ rot: dry matter 12 %. Slightly sticky.				
1	130	1.80	12.7	105.8
2	150	1.75	13.5	101.2
3	100	1.40	16.2	135.3
4	150	1.70	16.5	137.7
5	290	1.60	36.0	300.5
6	100	1.60	12.4	103.5
7	250	1.50	35.3	294.1
8	220	1.60	27.3	227.5
Mean				175.7
Standard deviation				85.4
Standard error of the mean				± 30.2

EXPERIMENTAL.

The physical test for stickiness was carried out on decomposed straws obtained under the various conditions outlined below.

Oat straw was rotted in presence of mixed flora for 30 days with the following changes in the environmental conditions.

- Variation in the initial moisture content at 35°.
- Variation in the sources of nitrogen at 35°.
- Variation in temperature—15°, 25°, 35° and 45°—with urea as the source of nitrogen.
- Variation in temperature—15°, 25°, 35° and 45°—with sodium nitrate as the source of nitrogen.
- Degree of decomposition at 35° with sodium nitrate as the source of nitrogen.
- Adjustment of p_H at 10.0 independent of the source of nitrogen.

Technique and methods.

Twenty g. of air-dry chaffed oat straw of known moisture and nitrogen contents were fermented aerobically in bottles with its natural mixed flora. Nitrogen was supplied to the extent of 1 g. per 100 g. of straw and the bottles incubated at the desired temperature. They were turned round in the first few days to ensure thorough wetting and frequently stirred to get homogeneous distribution of nitrogen and moisture. Water-logging was avoided as it causes anaerobic conditions. After the desired period each bottle was weighed with its contents and analysed as indicated below.

1. Extractions with water, 1 % sodium carbonate and 1 % sodium hydroxide. In each case 10 g. of the wet sample were boiled for 5 minutes with 100 cc. of water and filtered. The aqueous extract was evaporated to dryness on a water-bath and weighed. The alkaline extracts were precipitated with a few drops of hydrochloric acid, gently heated to coagulate the precipitate and then filtered through tared papers. The precipitates were well washed, dried and weighed.

2. Extractions with 90 % alcohol were carried out in a Soxhlet apparatus for 6 hours. Upon removal of the alcohol the extract was weighed, and after hydrolysis by boiling with 5 % H_2SO_4 for 2 hours its sugar content was determined by the Mohr-Bertrand method.

3. Treatment with hydrogen peroxide: the method employed was described by Shrikhande [1933].

4. Ammonia-N was determined by distillation with MgO .

5. Nitrate-N on the samples rotted with sodium nitrate was determined by distillation of the residue from ammonia-N in presence of alkali and Devarda's alloy.

6. Total N was determined by the usual Kjeldahl method. In the presence of nitrate-N the sulphuric-salicylic acid method was adopted.

RESULTS.

Series (a) and (b). Fungi were noticed on or about the 6th day. At the end of 30 days no trace of fungus mycelium was obvious except with ammonium carbonate and urea. *Coprinus* seemed to be very active in ammonium carbonate, urea and sodium nitrate rots. Better decomposition was observed with ammonium sulphate when calcium carbonate was introduced. Fungus growth was hardly noticeable at 15°. The rots appeared different under different conditions. The manures obtained, using ammonium carbonate, sodium nitrate and mould tissues as the source of nitrogen at high moisture contents, were noticeably slimy.

Effect of initial moisture content.

Table II gives the effect of initial moisture content upon the decomposition and nitrogen transformation. In each case the moisture content initially was adjusted at 60, 70, 80 and 90 %. During the course of decomposition an attempt was made to maintain the above levels of moisture but at the end of 30 days they seem to narrow down to about 85 to 98 %, which seems to be the optimum moisture necessary for pronounced rotting.

The series with ammonium carbonate, ammonium sulphate and sodium nitrate were repeated twice. Series (a) was used for nitrogen determinations and series (b) for extractions and the physical test for stickiness. The figures for moisture content and the losses of dry matter indicate that the higher the moisture content the greater the activity of the organisms and consequently the greater is the degree of decomposition. This observation agrees with that of Engberding [1909] who found an increased number of bacteria with the increased

Table II. *Nitrogen content of straws rotted by mixed floras with different sources of available nitrogen for 30 days at 35°.*

Expressed on 100 g. dry straw.							
Initial moisture %	Dry matter	Loss of D.M.	NH ₃ -N	NO ₃ -N	Total N	N factor	N equiv.
Ammonium carbonate.							
60	(a) 13.8 (b) 19.7	39.6 36.5	0.018	0.0	1.20	0.89	2.2
70	(a) 12.8 (b) 15.0	40.6 39.2	0.016	—	1.33	0.96	2.3
80	(a) 12.0 (b) 14.0	41.2 45.0	0.013	—	1.33	1.06	2.5
90	(a) 11.9 (b) 11.4	42.2 44.4	0.044	—	1.71	1.20	2.8
Ammonium sulphate.							
60	(a) 17.0 (b) 23.4	36.5 30.1	0.467	—	1.29	0.68	1.8
70	(a) 15.9 (b) 19.3	28.5 32.5	0.595	—	1.49	0.59	2.1
80	(a) 15.4 (b) 16.7	23.4 31.5	0.550	—	1.41	0.69	3.0
90	(a) 14.0 (b) 14.7	20.0 30.7	0.593	—	1.68	0.68	3.4
Sodium nitrate.							
60	13.8	30.7	0.041	0.119	1.76	0.83	2.7
70	(a) 13.6 (b) 15.2	49.2 52.8	0.012	0.020	1.18	0.81	1.6
80	(a) 13.4 (b) 13.3	43.7 52.3	0.009	0.009	1.20	0.63	1.4
90	12.6	48.7	0.027	0.027	1.31	0.89	1.8
Water.							
80	18.2	30.0	—	—	0.36	0.16	5.3
Peptone.							
75	16.5	39.5	0.004	—	1.48	1.12	2.8
75	15.6	44.1	0.007	—	1.49	1.10	2.4
Caseinogen.							
75	17.0	43.0	0.002	—	1.46	1.10	2.5
75	17.1	41.8	0.002	—	1.30	1.07	2.5
Urea.							
75	20.0	32.5	0.003	—	1.36	1.03	3.1
75	18.9	34.0	0.004	—	1.67	1.30	3.8

moisture content of the soil. There is, however, an exception to this in the case of ammonium sulphate where the order is reversed. It is possible therefore that a high moisture content with (NH₄)₂SO₄ as the source of nitrogen depresses the activity of the organisms.

The figures for "nitrogen factor" of straws rotted at 35° were determined for the sake of comparison and are given in column 7 of Table II. The nitrogen factor in these cases is the resultant of the many organisms involved and shows a tendency to increase with higher moisture content, meaning that it increases with the degree of decomposition for the particular source of nitrogen. Ammonium sulphate gives the lowest nitrogen factor whereas ammonium carbonate gives the highest with inorganic sources of nitrogen. Sodium nitrate

however does not appear so good as ammonium carbonate from the point of view of nitrogen immobilisation although it gives a higher decomposition. The nitrogen factor with organic sources of nitrogen is of little value because it is difficult to distinguish between nitrogen synthesised by the organisms and the organic nitrogen supplied.

"Nitrogen equivalents" are given in Table II, column 8. Sodium nitrate gives the lowest figure of 1.4 with 80 % initial moisture content indicating the greatest activity of the organisms per g. of N. The highest figure is 3.4 with $(\text{NH}_4)_2\text{SO}_4$ and 80 % moisture, no doubt due to the low activity of the organisms in this case.

Hutchinson and Clayton [1919], while discussing the decomposition of cellulose with *Spirochaete cytophaga*, say "from the chemical standpoint and on account of its insolubility in acids and solubility in ammonium hydroxide, the mucilage would without doubt appear in the 'crude humus' fraction in the conventional soil analysis." An attempt was made to extract this fraction possibly responsible for the stickiness. Table III gives the figures for various extracts and the values for the stickiness as measured by the physical test. The figures for stickiness were obtained on the mixture of samples with the different moisture contents for a particular source of nitrogen. The maximum stickiness was obtained with sodium nitrate, which also gave the greatest decomposition and the highest water, sodium carbonate and sodium hydroxide extracts. Mould tissue is the source of nitrogen which gives the maximum decomposition. 2 % nitrogen supplied as tissue gives extracts of the same order as sodium nitrate though the stickiness is rather lower. Double the amount of tissue produces more than double the amount of stickiness. This clearly indicates that stickiness depends a great deal upon the quantity of elaborated fungal tissue. The lowest figures for stickiness and extracts were obtained with ammonium sulphate. The best correlation between stickiness and extracts seems to be obtained with sodium carbonate extraction. This is expressed graphically in Fig. 2. The

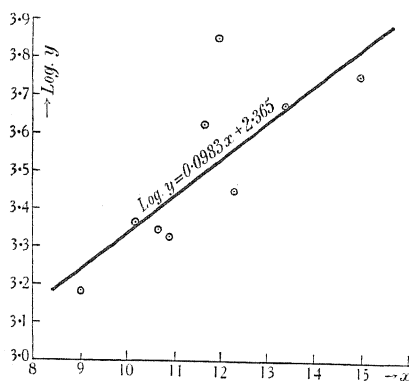


Fig. 2. Correlation between stickiness and sodium carbonate extracts of manures.

logarithm of stickiness when plotted against the extract can approximately be represented by a straight line. $\text{Log } y = 0.0983x + 2.365$.

The aqueous extract increases with the degree of decomposition. Water would extract some protein, very little of the hemicelluloses and any water-soluble material, gummy or otherwise, synthesised during decomposition. The larger water extract can therefore be explained by the presence of more of the

Table III. *Different extracts with physical test for stickiness and p_H values of straws rotted by mixed flora with different sources of nitrogen for 30 days at 35°.*

Expressed on 100 g. dry manure.

Initial moisture %	Loss of D.M.	Alcoholic extract	Sugars in alcoholic extract mg.	H ₂ O extract	NaOH extract (NH ₄) ₂ CO ₃ .	Na ₂ CO ₃ extract	Physical test g.	Initial p_H	Final p_H	H ₂ O ₂ extract
60	(a) 37.16 (b) 36.50	5.16 6.20	1139.5 —	— 21.1	— 16.9	— 11.5	4241	9.0	8.0	20.2
70	(a) 40.60 (b) 39.20	5.04 8.50	326.9 —	— 21.2	— 18.2	— 11.9				
80	(a) 41.20 (b) 45.00	4.30 5.20	462.5 —	— 22.2	— 11.4	— 8.8				
90	(a) 42.20 (b) 44.40	4.58 10.30	382.2 —	— 29.3	— 13.5	— 3.5				
60	(a) 36.50 (b) 30.10	4.19 9.00	418.0 —	— 17.9	— 16.8	— 7.7	220	5.5	6.5	14.5
70	(a) 38.50 (b) 32.50	4.45 8.20	529.0 —	— 20.5	— 13.3	— 4.1				
80	(a) 23.40 (b) 31.50	4.50 9.20	520.0 —	— 20.8	— 14.4	— 3.3				
90	(a) 20.0 (b) 30.7	5.30 9.30	513.0 —	— 19.8	— 12.2	— 4.5				
60	30.7	5.50	247.6	—	—	—	7164	6.35	10.0	27.4
70	(a) 49.2 (b) 52.8	3.80 9.10	104.8 —	— 44.3	— 17.4	— 15.5				
80	(a) 43.7 (b) 52.3	4.30 10.30	109.8 —	— 62.1	— 19.3	— 8.3				
90	48.7	4.35	182.0	—	—	—				
80	30.0	6.35	—	12.9	Water. 10.5	1.2	0	5.50	—	16.3
75	39.5	8.25	—	21.5	Peptone. 15.2	10.9	1504	5.75	7.5	16.3
75	44.1	7.11	—	26.9	16.6	7.2				
75	43.0	7.10	—	28.7	Caseinogen. 24.5	11.9	2727	—	7.5	15.8
75	41.8	11.05	—	27.2	19.4	13.5				
75	32.5	8.36	—	25.3	Urea. 19.7	11.6	2278	6.45	7.5	12.8
75	34.0	10.04	—	19.0	8.7	8.7				
80	35.9	—	—	—	(NH ₄) ₂ SO ₄ + CaCO ₃ .	10.6	2176	—	7.5	—
80	29.1	—	—	—	Ca(CN) ₂ .	13.2	4635	—	—	—
80	57.7	—	—	Mould tissue to give 1 % nitrogen. 28.4	10.3	17.9	2124	—	8.2	—
80	61.6	—	—	Mould tissue to give 2 % nitrogen. 36.9	10.0	20.3	5622	—	8.2	—

protein and other microbial constituents which are progressively synthesised and possibly also of more gummy material which seems to increase, as indicated by the high stickiness with the physical test.

The alkaline extracts diminish with the time of decomposition. Alkaline treatment extracts more than water alone, but only a part of the substances in solution is precipitated by acid. The materials extracted with alkali are

practically of a similar nature to those obtained with hot water. Extraction may vary with increasing concentration and nature of the alkali. 1 % sodium hydroxide would extract lignin to an appreciable extent, and on comparing the figures for sodium hydroxide and sodium carbonate extracts it is seen that the sodium hydroxide extract is nearly one and a half times the sodium carbonate extract. The weight of the extracts increases as the stickiness increases.

Of organic solvents only alcohol was found to extract a certain fraction of manure and the humus from it. Alcoholic extracts are practically of the same order in each case. There is a tendency for the extract to increase with the degree of decomposition. The sugar content or the extract from ammonium carbonate on an average is greater than that from ammonium sulphate or sodium nitrate.

The significance of extraction with hydrogen peroxide is discussed in detail elsewhere [Shrikhande, 1933]. Much importance cannot be attached to the relationship between peroxide extract and stickiness. The nature of this extraction differs from others in the sense that this solvent oxidises some of the decomposed and synthesised material. All extracts if plotted graphically against stickiness give a straight line. In the rots with caseinogen there is an exception to the general order as mentioned above, all the extracts being disproportionately greater. This may be due to the fact that less caseinogen is decomposed by the organisms, and the remainder is dissolved by the solvents and returned unchanged in precipitates.

The initial p_H was highest with ammonium carbonate and lowest with ammonium sulphate. Sodium nitrate which was practically neutral at the start gave a final p_H of 10.0. The highest stickiness and decomposition were obtained with sodium nitrate and correspondingly lowest figures with ammonium sulphate, which maintained an acid reaction throughout. When, however, an equivalent amount of calcium carbonate was supplied to the ammonium sulphate rot, decomposition increased with an increase in stickiness. This leads to the conclusion that maximum stickiness is associated with alkaline conditions. This may mean either that the organisms responsible for stickiness are favoured by an alkaline medium, that there is a modification of the flora with the change in the environmental conditions or that the manifestation of the property of stickiness is enhanced by alkaline conditions. The p_H with all the three organic sources of nitrogen is practically of the same order (7.5) and similar figures for stickiness were obtained. The final high p_H with sodium nitrate is no doubt due to the utilisation of nitrate-N leaving excess of base. The slight lowering of p_H with ammonium carbonate may be ascribed to the use of nitrogen from ammonia and the liberation of CO_2 .

Table IV. *Different extracts with physical test for stickiness and final p_H values of straws rotted by mixed floras with available nitrogen as urea and $NaNO_3$ at different temperatures.*

Expressed on 100 g. dry manure.							
Temp. ° C.	Source of N	Loss of D.M.	Physical test (g.)	Final p_H	Water extract	Na_2CO_3 extract	NaOH extract
15	Urea	7.2	414	7.8	17.1	7.7	13.2
	$NaNO_3$	12.3	2610	9.0	23.2	5.2	10.5
25	Urea	40.6	5020	8.0	29.6	11.9	16.0
	$NaNO_3$	30.2	3678	9.5	31.8	8.5	17.5
35	Urea	33.0	2278	7.5	25.3	11.6	19.7
	$NaNO_3$	52.5	7164	10.0	44.3	15.5	19.3
45	Urea	58.2	3650	8.5	38.7	18.1	19.8
	$NaNO_3$	40.9	7875	10.0	47.5	18.4	22.3

Series (c) and (d). Variation in temperature with urea and sodium nitrate as sources of nitrogen. Table IV contains results of extraction, final p_H and stickiness on samples of straws obtained as indicated above.

The losses of dry matter seem to increase with the increase in temperature. At 15° the decomposition is very poor in both cases, though sodium nitrate gives double that of urea. The decomposition obtained with urea at 25° is more than that at 35°. At 45° urea gives a decomposition of 58 %, which is much more than with sodium nitrate. These variations clearly indicate the different nature of the flora working at the different temperatures. The final p_H for urea varies between 7.8 at 15° and 8.5 at 45°. Similarly the p_H with sodium nitrate has increased from 9.0 at 15° to 10.0 at 45°. The initial p_H values were 6.45 and 6.35 respectively.

Stickiness. Even with a very small decomposition sodium nitrate produces quite an appreciable amount of stickiness, which increases with the rise in temperature. The maximum stickiness with urea as a source of nitrogen is produced at 25°.

Series (e). Degree of decomposition at 35° with sodium nitrate as the source of nitrogen. Table V indicates the relationship observed between the stickiness of the manure and the degree of decomposition when sodium nitrate was supplied

Table V. *Effect on the production of stickiness of modification of the p_H and the degree of decomposition.*

Source of N	Time in days	Loss of D.M. %	Physical test in g.					
			On manure	On original straw	Final p_H	p_H adjusted		On manure
						To	With	
NaNO ₃	8	4.7	2050	1954	8.0	9.5	Na ₂ CO ₃	3868
"	16	23.1	4020	3903	9.0	9.5	Na ₂ CO ₃	5521
						9.5	K ₂ CO ₃	5123
						8.0	H ₂ SO ₄	3648
						5.5	H ₂ SO ₄	2305
"	24	32.2	7834	5310	9.5	—	—	—
"	32	34.8	8161	5320	9.5	8.0	H ₂ SO ₄	5991
						7.0	H ₂ SO ₄	4538
						5.5	H ₂ SO ₄	3410

as the source of nitrogen. The physical test increases markedly as decomposition proceeds. There was, however, a possibility that this might be an effect of the changing reaction of the rot, which becomes progressively more alkaline and reaches finally a p_H of 9.5. To investigate this point the samples after 8 and 16 days' decomposition were adjusted to the final p_H of 9.5 by addition of sodium carbonate. This had the effect of increasing the figures for the physical test very appreciably, but not so much that they approached the level obtained at this p_H with longer periods of decomposition. There is therefore a direct relationship between the degree of decomposition of a manure and its stickiness, even when all the changes in the reaction have been taken into account. This point is more clearly brought out when the physical test is recalculated on a basis of original straw. The stickiness increased till the 24th day, after which further loss of organic matter was not accompanied by further production of apparent sticky material.

Further to demonstrate the effect of reaction, samples rotted for 32 days, having achieved the very high degree of stickiness indicated by a physical test of 8161 g., were acidified and the p_H reduced to 8.0, 7.0 and 5.5. This had the effect

of lowering the stickiness to 5970 g., 4538 g. and 3410 g. respectively. Similar adjustments of the p_H with sodium carbonate, potassium carbonate and sulphuric acid were made with the sample rotted for 16 days. These experiments indicate that the stickiness varies directly with the p_H within the limits tested. The relation between stickiness and reaction is represented graphically in Fig. 3.

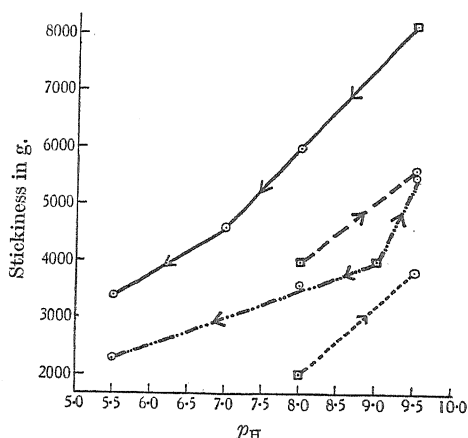


Fig. 3. Relationship between stickiness and reaction.

□ Initial point. → Changes due to alteration in p_H .
 — NaNO_3 30 days. — Urea 30 days.
 - - - NaNO_3 16 days. . . . NaNO_3 8 days.

Series (f). Adjustment of p_H at 10.0 independently of the source of nitrogen. Table VI deals with the effect on stickiness of the initial reaction of the material undergoing decomposition. Various sources of nitrogen were supplied and the

Table VI. Effect of the initial p_H on the production of stickiness.

Source of N	Initial p_H brought to 10.0 with	Final p_H	Loss of D.M. %	Physical test g.
Peptone	MgCO_3	8.0	35.3	3938
Peptone	Na_2CO_3	8.5	49.0	6490
Mould tissue as 1 % N	MgCO_3	8.0	37.0	4789
*Mould tissue as 1 % N	Na_2CO_3	8.0	52.2	4114
Mould tissue as 2 % N	MgCO_3	8.0	50.3	4863
Mould tissue as 2 % N	Na_2CO_3	8.5	56.5	5349
Urea	CaCO_3^\dagger	8.0	39.0	3991
Urea	Na_2CO_3	8.5	42.3	6001

* Water-logged.

† Final p_H brought to 10.0 by adding Na_2CO_3 .
 Stickiness then equals 5620 g.

initial p_H of the straw adjusted to 10.0 with magnesium carbonate in one series and sodium carbonate in another. This particular reaction was chosen because the highest figure hitherto obtained for stickiness was found in a rot supplied with sodium nitrate which had attained finally that high degree of alkalinity. During fermentation the p_H values of both the series fell somewhat, those with magnesium carbonate more than those with sodium carbonate. The losses of dry matter and stickiness obtained were however invariably greater in the latter series. It is clear that if rots are adjusted initially to a high degree of

alkalinity the manure resulting is stickier than that which is usually obtained. Furthermore it appears that, given the correct p_H , sodium or potassium ions are more favourable than are calcium and magnesium.

The effect of sticky manure on soils.

Three soils of different composition were selected, a Rothamsted soil which owing to a high clay content is very heavy, a Woburn and a Cheshire soil, both of which are light, but the former more sandy. Two grams of the soil sample were mixed as uniformly as possible with one gram of the sticky manure and made into a paste with water. The dry matter of the mixed sample was adjusted to about 50 %. The physical test was carried out on one gram of this mixture as in the case of manures. Stickiness of the soils when wet and after drying was also determined to compare with the figures after mixing them with the sticky manure. The following table contains these figures.

Soil	Physical test in g.		
	Wet	Dried	When mixed with manure and dried
Rothamsted	431	924	947
Cheshire	0	424	829
Woburn	0	214	686

There is therefore a definite increase in the stickiness of light soils on mixing with such a manure.

The nature of the mucus.

Attempts to extract the sticky constituents of the manure with different solvents give unsatisfactory results. The usual method of precipitating gums from water and mildly alkaline extracts with absolute alcohol and Fehling's solution was also tried without success.

A sodium nitrate rot with mixed flora, which was definitely sticky to the touch and markedly so by the physical test, was extracted with cold water. The extract was colloidal. It was filtered twice through glass wool and finally through a filter-paper under suction. The extract was then precipitated with a few drops of HCl. The precipitate was coagulated by gentle heating on a hot plate and then filtered on a Büchner funnel. A very small fraction of this mixed with 1 g. of dry oat straw proved to be quite sticky. On drying the precipitate became very hard and gritty and lost its binding properties in part. Having thus established that the above precipitate contains a sticky constituent both when wet and dry, the following analytical figures are obtained on the extract.

Water extract	9.86 % on dry matter
Physical test for stickiness with 1 g. of the wet manure	4880 g.
Physical test with the wet extract	2555 g.
Physical test with the extract dried and then re-moistened	1575 g.
On hydrolysis with 3 % H_2SO_4 for 5 hours				
Apparent anhydroglucose	52.04 %
Anhydropectose	2.46 %
Protein	21.50 %
Ash	3.20 %
			Total	79.20 %

After acid hydrolysis the extract reduced Fehling's solution and gave Selivanoff's test for fructose. On oxidation of a portion with concentrated nitric acid a precipitate was obtained consisting of colourless plates and micro-sandy crystals. The crystals were separated by shaking up with hot alcohol in which the plates dissolved. The insoluble residue had m.p. over 200°, and when distilled with conc. HCl yielded furfuraldehyde indicating the presence of mucic acid.

The extract appears therefore to be a mixture of carbohydrates and proteins, and no doubt in part consists of material extracted from the elaborated microbial tissue. The carbohydrate portion of the extract seems to consist largely of galactan, though indications have been obtained also of the presence of uronic acids and a little pentose.

SUMMARY.

1. The conditions under which stickiness is produced in decomposing plant materials and manures have been investigated and some information obtained as to the nature of the substances contributing this property.
2. A physical test for evaluating the property of stickiness in manures has been described.
3. In the presence of a mixed natural flora, the chief factors involved in causing stickiness in decomposing straw are the source of nitrogen supplied, the initial and final reactions of the material and the degree of decomposition.
4. High values for stickiness are given with either sodium nitrate or mould tissues as the sources of nitrogen. This suggests that an alkaline reaction and an abundance of microbial tissue are essential in the production of stickiness during decomposition by mixed flora.
5. The final reaction of the manure profoundly influences the degree of stickiness, if at all appreciable. A p_H of 9.5 to 10.0, whether obtained by fermentation or by subsequent adjustment, seems to give the maximum stickiness. Sodium or potassium ions produce more stickiness than calcium or magnesium.

The author is indebted to Sir John Russell, Director of the Rothamsted Experimental Station, for placing at his disposal the facilities of the Station, and to Mr E. H. Richards, Head of the Fermentation Department, for suggesting the problem and for invaluable advice and criticism. Thanks are due to Dr A. G. Norman for his assistance and suggestions, and to Mr Scott Blair, of the Physics Department, for criticising the physical test.

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CCXIV. THE PRODUCTION OF MUCUS DURING THE DECOMPOSITION OF PLANT MATERIALS¹.

II. THE EFFECT OF CHANGES IN THE FLORA.

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(Received July 27th, 1933.)

THE production of stickiness during the process of decomposition of cellulosic materials has been attributed by Hutchinson and Clayton [1919] to the activity of soil organisms such as *Spirochaeta cytophaga*. Since the time of Mitscherlich [1850] bacteria were considered to be the chief agents in the natural decomposition of plant materials until König [1904] suggested the greater importance of fungi. McBeth and Scales [1913] and Scales [1915] found fungi to be capable of using cellulose as the sole source of energy. It has been shown by Waksman [1926] and later confirmed by Norman [1929] that in the presence of sufficient available nitrogen, cellulose is the chief constituent to be decomposed. Waksman [1924] and Rege [1927] have shown that fungi are mainly responsible for the decomposition especially during the early stages.

Norman [1931] has studied the nitrogen transformations and changes in the carbohydrate constituents with pure cultures of fungi but no such study has so far been made of the subsequent action of bacteria after fungal attack, or of the simultaneous action of a bacterium with that of a fungus. Apart from the study of stickiness produced during decomposition, it was thought worth while to follow the ammonification and nitrogen changes and the losses in carbohydrate constituents at different stages of decomposition.

The work described in this paper was carried out in order to test whether mucus is produced during decomposition of straw with pure cultures of organisms. Straw was therefore decomposed with selected fungi, fungi and bacteria and bacteria alone. Analyses of the different constituents were made in order to find any correlation between the rates of their decomposition and the formation of mucus. The amount of mucus produced was measured by the physical test described in Part I [1933].

Scheme of work.

(1) Oat straw was rotted with pure cultures of fungi at 35° with ammonium carbonate as source of nitrogen and analysed at intervals of 8, 16, 24 and 48 days.

(2) Straw was rotted with pure cultures of bacteria: (i) *Mycobacterium agreste*, (ii) *Spirochaeta cytophaga*, and analysed at intervals of 8, 16, 24 and 48 days to see if any bacterium which, like the latter, produces a gummy colony on an artificial medium would produce stickiness in straw.

(3) Straw was rotted first for 48 days with fungi alone: (a) and then inoculated with *M. agreste* and analysed on the 8th, 16th, 24th and 48th days after inoculation; (b) as (a) but with the substitution of *S. cytophaga* for *M. agreste*.

¹ This paper is an abridged form of part of a thesis approved for the Degree of Doctor of Philosophy in the University of London.

(4) Straw was rotted with a fungus progressively and then inoculated with *S. cytophaga* on the 8th, 16th, 24th and 48th days, and analysed on the 8th and 40th days after inoculation with the latter.

(5) Straw was rotted simultaneously with fungus and *S. cytophaga* and analysed on the 8th, 16th, 24th and 48th days.

Methods.

Weighed quantities of straw were bottled and sterilised in an autoclave under 115 lbs. pressure for 45 minutes on two consecutive days. The treatment is undoubtedly drastic yet it is necessary for complete sterilisation. Available nitrogen was supplied as sterile ammonium carbonate in the proportion of 1 g. per 100 g. straw and the moisture was adjusted at about 80 %. A heavy inoculum of a suspension of the spores of the required organism in sterile water was then added to each bottle. Platings were made at the end of the experiments to test the purity of the organism. Fungal contamination was easily noticeable in most cases, since the straw was characteristically coloured by the spores of the fungi.

The following fungi were tested: *Trichoderma lignorum*, *Acremonia olivacea*, *Aspergillus niger*, *A. terreus*, *A. nidulans*.

The fungi tested were obtained from the stock cultures of the Mycology Department (isolated from the Rothamsted soils). They were maintained on agar slants in Waksman's medium. Before inoculation they were always grown afresh and in all the experiments described below cultures one week old were used.

Estimations of ammonia-N, total N, extraction with 90 % alcohol and treatment with hydrogen peroxide were carried out as in Part I.

(1) *Pentose units* (total furfuraldehyde). The standard method of Krobe and Tollens was employed. The phloroglucide precipitate was not extracted with alcohol.

(2) *Cellulose* was estimated by Jenkins's method [1930].

(3) *Furfuraldehyde in cellulose* was determined as in (1).

The loss of dry matter (D.M.) as a result of decomposition is usually not a true index of the amount of decomposition. It does not consider the synthesis of new complexes by micro-organisms, which would modify to a considerable extent the actual amount of material decomposed. The cellulose and furfuraldehyde determinations would indicate how much of the loss in organic matter is represented by the loss in cellulose and hemicelluloses.

In any mature plant material, the cellulose, the polysaccharide associated with cellulose, or as it is termed by Hawley and Norman [1932], the "cellulosan," and the hemicelluloses are the three chief groups of biologically available material. The cellulose as isolated either by the Cross and Bevan chlorination method or by the Jenkins hypochlorite method is always associated with cellulosan. This cellulosan generally consists of pentose units. In the case of oat straw it is xylan as proved by Norman [1929]. This xylan can be determined by distilling the cellulose with 12 % HCl. A figure for "true" cellulose can then be obtained by deducting the figure for xylan. It is found that the decompositions of cellulose and the xylan associated with it run parallel. A figure for pentose units in the hemicelluloses can be obtained by subtracting from the total yield of furfuraldehyde that from the xylan associated with cellulose.

For the sake of comparison the figures recorded below are calculated on a basis of 100 g. of dry straw and interpreted in terms of cellulose, xylan associated with cellulose and pentose in hemicelluloses.

EXPERIMENTAL.

The same sample of oat straw was used throughout these experiments with pure cultures of organisms.

	%
Ash	9.04
Total N	0.35
Total furfuraldehyde	15.6
"True" cellulose	41.0
Furfuraldehyde on Jenkins's cellulose product	7.0
Xylan associated with cellulose	10.8
Furfuraldehyde due to pentose in polyuronides	8.6
Alcohol extract	3.46

Series I (Tables I and II).

Stickiness. No stickiness was observed with any fungi used for decomposition.

Carbohydrate constituents. Table I contains analyses of these constituents. *Acremonia* sp. appears to be the most active fungus as judged from the loss of dry matter at the end of 48 days. *T. lignorum* seems to be the least active. The losses obtained follow practically the same order as those recorded by Norman [1931]. In general the hemicelluloses suffer a loss of about 60 % during 48 days. The hemicelluloses appear to decompose rapidly in the first few days and then remain at much the same level while the cellulose is being decomposed. On the whole it is obvious that the major part of the lost carbonaceous material is accounted for by the cellulose which is a more abundant food and energy source for the micro-organisms than the hemicelluloses.

Table I. *Decomposition of straws by various fungi at 35° at different intervals.*

Fungus	Time in days	Expressed on 100 g. original straw.				Loss of furfuraldehyde from non-cellulosic constituents
		Loss of D.M.	Furfuraldehyde from pentose groups not in cellulose	"True" cellulose	Loss of "true" cellulose	
<i>Acre. olivaceospora</i>	8	10.9	6.37	36.90	4.1	2.23
	16	21.2	5.19	27.81	13.1	3.41
	24	28.1	4.56	23.65	17.3	4.04
	48	41.2	3.32	16.17	24.8	5.28
<i>A. nidulans</i>	8	9.2	7.04	38.85	2.1	1.56
	16	15.6	6.01	35.64	5.3	2.59
	24	31.5	3.87	22.42	18.5	4.73
	48	38.9	3.23	17.02	23.9	5.37
<i>A. terreus</i>	8	11.0	6.98	37.66	3.3	1.62
	16	15.9	5.22	31.23	9.7	3.38
	24	31.9	4.66	24.09	16.9	3.94
	48	34.6	4.01	19.88	21.1	4.59
<i>A. niger</i>	8	11.2	4.98	37.37	2.6	3.62
	16	15.8	5.38	34.27	7.1	3.22
	24	23.1	4.86	26.25	14.7	3.74
	48	34.1	3.67	23.13	17.8	4.93
<i>T. lignorum</i>	8	9.3	4.23	38.29	2.7	4.37
	16	16.0	5.08	35.08	5.9	2.80
	24	24.4	5.02	27.82	13.1	3.58
	48	29.0	4.17	23.36	16.6	4.43

Table II. Nitrogen content of straws rotted by pure cultures of fungi at 35° at different intervals.

Expressed on 100 g. original straw.

Fungus	Time in days	Loss of D.M.	Total N	Organic N	N factor	N equiv.
<i>Acre. olivaceospora</i>	8	10.9	1.37	0.98	0.63	5.7
	16	21.2	1.32	1.29	0.93	4.4
	24	28.1	1.24	1.21	0.85	3.0
	48	41.2	1.19	1.17	0.84	2.0
<i>A. nidulans</i>	8	9.2	1.14	0.62	0.27	2.9
	16	15.6	0.92	0.72	0.37	2.3
	24	31.5	0.94	0.92	0.56	1.8
	48	38.9	1.12	1.09	0.73	1.8
<i>A. terreus</i>	8	11.0	1.22	0.75	0.40	3.7
	16	15.9	1.12	0.99	0.64	4.0
	24	31.9	0.85	0.82	0.51	1.6
	48	34.6	1.02	1.01	0.66	1.9
<i>A. niger</i>	8	11.2	1.20	0.82	0.46	4.1
	16	15.8	1.11	1.03	0.65	4.1
	24	23.1	1.24	1.22	0.86	3.7
	48	34.1	1.02	1.02	0.86	2.5
<i>T. lignorum</i>	8	9.3	1.23	0.86	0.50	5.4
	16	16.0	1.09	0.93	0.65	4.8
	24	24.4	1.25	1.24	0.88	3.6
	48	29.0	1.25	1.24	1.01	3.4

Nitrogen immobilisation. From Table II it can be seen that more than 50 % of the inorganic nitrogen has been used up by the organisms except *A. nidulans* in the first 8 days. The power of an organism in building up the microbial protein can best be judged from the nitrogen factor [Richards and Norman, 1931]. *T. lignorum* seems to be the best from the point of view of nitrogen immobilisation and *A. nidulans* the poorest. Norman [1931] also recorded a low nitrogen factor for *A. nidulans* compared with *A. niger* and *A. terreus*. As compared by their nitrogen equivalents [Norman, 1931] *A. nidulans* seems to be the most efficient and *Trichoderma* the least. *A. niger* differs markedly from *A. nidulans* and *A. terreus* though belonging to the same genus.

Series II (Tables III, IV and V).

Stickiness. Both the organisms produce a negligible amount of stickiness. The figures for *S. cytophaga* are contrary to expectation. Since it is an organism which produces a gummy colony on agar media, it was supposed that it might also give a rotted product showing stickiness.

Table III. Physical test, alcoholic extracts and the H₂O₂ treatment of straws rotted with pure cultures of bacteria at 35° at different intervals.

Expressed on 100 g. dry manure.

Bacterium	Time in days	Loss of D.M.*	Alcohol extract	H ₂ O ₂ extract	Loss of O.M. after H ₂ O ₂ treatment	Physical test (g.)
<i>S. cytophaga</i>	8	7.5	8.40	7.0	10.1	108.6
	16	12.2	6.87	10.1	11.4	108.2
	24	13.0	7.48	13.9	11.4	212.3
	48			Infected		
<i>M. agreste</i>	8	4.8	7.05	13.7	12.6	101.3
	16	8.5	7.41	12.1	11.4	0.0
	24	14.2	7.51	9.7	13.8	334.1
	48			Infected		

* On 100 g. original straw.

Extractions with alcohol and hydrogen peroxide (Table III). On an average the alcoholic extract is the same for both of the organisms with no further change in the degree of decomposition. There is an increase in the hydrogen peroxide extract with *S. cytophaga* whereas the order is reversed with *M. agreste*.

Carbohydrate constituents (Table IV). The decomposition is practically of the same order with both the organisms at the end of 24 days. The bottles for the 48th day were infected and hence rejected. The cellulose lost with *Mycobacterium* is nearly one and a half times that destroyed by *Spirochaeta*. This was

Table IV. *Decomposition of straw by bacteria at 35° at different intervals.*

Expressed on 100 g. original straw.						
Bacterium	Time in days	Loss of D.M.	Furfuraldehyde from pentose groups not in cellulose	"True" cellulose	Loss of "true" cellulose	Loss of furfuraldehyde from non-cellulosic constituents
<i>S. cytophaga</i>	8	7.5	5.60	38.82	2.1	3.00
	16	12.2	6.11	36.76	4.2	2.59
	24	13.0	5.31	36.29	4.7	3.29
	48			Infected		
<i>M. agreste</i>	8	4.8	6.06	39.14	1.8	2.54
	16	8.5	6.02	36.43	4.5	2.58
	24	14.2	5.95	34.31	6.6	2.65
	48			Infected		

entirely unexpected, since the former is not ordinarily regarded as being a cellulose-decomposing organism and does not develop on a cellulose-agar plate, but, as pointed out by Norman [1930], the ability to utilise cellulose in the presence of other available carbohydrates is more common than is generally supposed. On the other hand *Spirochaeta* destroys hemicelluloses to a considerably greater extent than does *Mycobacterium*. Both organisms compare poorly with fungi in destructive action as regards organic matter.

Nitrogen immobilisation. The nitrogen figures are recorded in Table V. *Mycobacterium* builds up more microbial tissue than does *Spirochaeta* as seen

Table V. *Nitrogen content of straw rotted with pure cultures of bacteria at 35° at different intervals.*

Expressed on 100 g. original straw.						
Bacterium	Time in days	Loss of D.M.	Total N	Organic N	N factor	N equiv.
<i>S. cytophaga</i>	8	7.5	1.268	0.75	0.39	5.2
	16	12.2	1.000	0.64	0.29	2.3
	24	13.0	0.957	0.80	0.46	3.5
				Infected		
<i>M. agreste</i>	8	4.8	1.290	0.93	0.58	11.8
	16	8.5	1.040	0.66	0.30	3.5
	24	14.2	1.180	0.97	0.60	4.2
				Infected		

from the nitrogen factor. Compared with fungi, bacteria appear to be much slower in building up microbial protein. *Spirochaeta* consumes more carbohydrate per g. of nitrogen than *Mycobacterium* as shown by the nitrogen equivalent.

Table VI. *Decomposition of straw rotted first with pure cultures of fungi for 48 days and then inoculated with M. agreste and analysed at different intervals.*

Expressed on 100 g. original straw.

Fungus	Days after inoculation	Loss of D.M.	Furfuraldehyde from pentose groups not in cellulose	"True" cellulose	Loss of "true" cellulose	Loss of furfuraldehyde from non-cellulosic constituents
<i>T. lignorum</i>	8	30.1	3.52	24.74	16.2	5.08
	16	44.5	2.34	12.92	28.0	6.26
	24	49.1	2.46	11.86	29.1	6.14
	48	39.0*	3.61	21.14	19.8	5.00
<i>A. niger</i>	8	35.7	3.74	20.20	20.8	4.86
	16	42.0	3.04	15.99	25.0	4.56
	24	36.0	3.63	17.52	23.4	4.97
	48	41.0	3.06	17.70	23.3	4.74
<i>A. nidulans</i>	8	25.0	4.39	29.53	11.4	4.21
	16	28.0	4.26	27.38	12.6	4.34
	24	36.0	2.94	18.98	22.0	5.66
	48	38.7	3.79	20.96	20.0	4.81
<i>Acre. olivaeformis</i>	8	32.4	4.06	25.18	15.8	4.54
	16	43.3	3.14	16.48	24.5	5.46
	24	43.4	2.94	15.59	25.4	5.66
	48	44.5	3.60	18.32	22.6	5.00
<i>A. terreus</i>	8	31.2	3.57	23.12	17.8	5.03
	16	36.9	3.55	19.00	22.0	5.03
	24	37.5	3.91	21.19	19.8	4.69
	48	39.7	3.42	19.81	21.2	5.18

* Short of moisture.

Table VII. *Nitrogen content of straw rotted first with pure cultures of fungi for 48 days and then inoculated with M. agreste and analysed at different intervals.*

Expressed on 100 g. original straw.

Fungus	Days after inoculation	Loss of D.M.	Total N	Organic N	N factor	N equiv.
<i>T. lignorum</i>	8	30.1	1.01	1.00	0.58	3.3
	16	44.5	1.22	1.21	0.70	1.5
	24	49.1	1.14	1.10	0.77	1.5
	48	39.0*	0.91	0.88	0.55	1.4
<i>A. niger</i>	8	35.7	1.08	1.06	0.72	2.0
	16	42.0	1.25	1.19	0.84	2.0
	24	38.0	0.95	0.94	0.67	1.7
	48	41.0	0.92	0.90	0.54	1.3
<i>A. nidulans</i>	8	25.0	0.97	0.95	0.62	2.5
	16	28.4	0.97	0.96	0.60	2.1
	24	36.9	1.16	1.14	0.80	2.1
	48	38.7	0.97	0.95	0.60	1.5
<i>Acre. olivaeformis</i>	8	32.4	1.00	0.98	0.70	2.1
	16	43.3	1.25	1.19	0.84	1.9
	24	43.4	1.19	1.13	0.82	1.9
	48	44.5	1.10	1.08	0.73	1.6
<i>A. terreus</i>	8	31.2	1.06	1.05	0.69	2.2
	16	36.9	0.97	0.96	0.59	1.6
	24	37.5	0.87	0.86	0.51	1.3
	48	39.7	0.95	0.88	0.53	1.3

* Short of moisture.

Series III (a) (Tables VI and VII).

Stickiness. The rots showed no stickiness, indicating that *M. agreste* is unable to synthesise sticky material even after acting upon the elaborated fungal tissue.

Carbohydrate constituents (Table VI). *M. agreste* following upon *Trichoderma* gives the best decomposition and a loss of 49.1 % organic matter in only 24 days. The losses with the *Aspergilli* are practically of the same order. The rate of decomposition in the first eight days after inoculation with all the fungi is greater than in any subsequent periods. The average loss of cellulose with *Mycobacterium* following upon the five different fungi ranges from 20 to 23 %. Further decomposition does not affect the pentose unassociated with cellulose showing that all the available pentoses have already been removed by the fungi.

Nitrogen immobilisation (Table VII). With *Mycobacterium* following on each of the fungi, there is a drop in the nitrogen factor after the 16th day in general, which is due to the ammonification or as it is termed by Jensen [1929] the "mineralisation" of a portion of the fungal protein.

Series III (b) (Tables VIII, IX and X).

Stickiness. The results for stickiness are given in Table VIII and are highly significant. No stickiness was found with fungi on the 48th day, but by the subsequent action of *S. cytophaga* for 8 days only, the pull required to separate

Table VIII. *Physical test, alcohol extracts and the H₂O₂ treatment of straws rotted first with pure cultures of fungi and then inoculated with S. cytophaga and analysed at different intervals.*

Expressed on 100 g. dry manure.						
Fungus	Days after inoculation	Loss of D.M.	Alcohol extract	H ₂ O ₂ extract	Loss of o.m. with H ₂ O ₂	Physical test (g.)
<i>T. lignorum</i>	8	39.2	10.50	13.1	22.0	3495
	16	39.7	8.50	12.1	22.3	6435
	24	42.6	8.87	12.8	23.0	3958
	48	57.7	12.20	29.0	26.0	6550
<i>A. niger</i>	8	41.4	9.90	13.3	24.7	6305
	16	45.0	8.90	17.5	25.6	4242
	24	46.0	10.20	26.6	24.7	7134
	48	48.3	9.20	24.7	25.0	6145
<i>A. nidulans</i>	8	35.8	8.80	11.7	22.8	5197
	16	41.2	8.60	16.8	26.2	6796
	24	43.4	9.04	13.8	30.0	5869
	48	43.5	9.05	22.8	22.0	5290
<i>Acre. olivaceospora</i>	8	43.1	9.14	13.3	24.0	4425
	16	43.2	8.02	13.8	23.2	7866
	24	45.0	8.56	21.0	25.0	4995
	48	46.3	7.64	15.0	25.0	6904
<i>A. terreus</i>	8	37.5	7.64	14.2	23.7	6439
	16	41.9	7.90	11.8	26.4	5970
	24	45.4	7.64	15.0	25.0	8386
	48	51.3	7.82	26.4	25.0	6442

The final p_H of all the samples was between 7.0 and 7.5.

the two plates was at once raised by from 3 to 6 kg. varying with the nature of the fungus. This obviously means that *Spirochaeta* has synthesised some organic matter which is responsible for the stickiness. Since it was observed in Series II that *Spirochaeta* alone could hardly produce any stickiness, one is naturally led

to believe that the sticky material has some relation to the previous action of the fungus. Again, *Mycobacterium* did not produce any stickiness even after a previous fungal decomposition, suggesting that the production of stickiness has also some relation to the nature of the bacterium taking part in the decomposition.

Extractions by alcohol and hydrogen peroxide (Table VIII). The averages for the alcohol extracts differ in each case, the maximum being 10 % with *Spirochaeta* following on *Trichoderma* and 7.7 % on *A. terreus*. These figures are interesting when compared with stickiness. The maximum stickiness obtained is with the latter combination, whereas the former gives the least. On the whole the alcoholic extracts are greater than those with *Mycobacterium*. The higher solubility of the sticky material in alcohol indicates its possible non-gummy and non-dextrin nature. The losses of organic matter on treatment with peroxide are in general proportional to the losses in dry matter.

Carbohydrate constituents (Table IX). Greatest decomposition is obtained by *Spirochaeta* following on *Trichoderma* and *A. terreus* and amounts to 51.7 and 51.3 % respectively. In these two cases there is a big jump in the loss of dry

Table IX. *Decomposition of straws rotted first with pure cultures of fungi for 48 days and then inoculated with S. cytophaga and analysed at different intervals.*

Expressed on 100 g. original straw.					
Fungus	Days after inoculation	Loss of D.M.	Furfuraldehyde from pentose groups not in cellulose	"True" cellulose	Loss of furfuraldehyde from non-cellulosic constituents
<i>T. lignorum</i>	8	39.2	3.16	14.61	5.24
	16	39.7	3.06	18.26	5.54
	24	42.6	3.17	14.89	5.43
	48	51.7	2.21	10.58	6.39
<i>A. niger</i>	8	41.4	2.88	14.57	5.72
	16	45.0	2.44	13.78	6.16
	24	46.0	2.56	13.52	6.04
	48	48.3	2.39	12.03	6.21
<i>A. nidulans</i>	8	35.8	3.88	18.46	4.72
	16	41.2	2.89	13.65	5.71
	24	43.4	3.09	16.28	5.51
	48	43.5	2.94	13.89	5.66
<i>Acre. olivaceospora</i>	8	43.1	2.86	13.79	5.74
	16	43.2	3.41	15.85	5.19
	24	45.0	2.66	13.42	5.94
	48	46.3	2.20	13.06	6.40
<i>A. terreus</i>	8	37.5	3.15	16.45	5.45
	16	41.9	2.86	14.61	5.74
	24	45.4	2.20	13.74	6.40
	48	51.3	2.06	12.58	6.54

matter between the 24th and 48th days after the inoculation with *Spirochaeta*, but with the remainder the decomposition appears to be steady. The average loss of cellulose is about 26 % in each case. The average loss of pentose not associated with cellulose is also practically the same. These losses are not significantly higher than those given by fungi alone at the end of 48 days, indicating that no available hemicelluloses are left after fungus action.

Nitrogen immobilisation (Table X). Here again there is a drop in the nitrogen factor as the decomposition proceeds. This fall is more marked with *Spirochaeta* than with *Mycobacterium*. The former appears more efficient than the latter per unit of nitrogen as judged by the nitrogen equivalent.

Table X. *Nitrogen content of straws rotted first with fungi for 48 days and then inoculated with S. cytophaga and analysed at different intervals.*

Expressed on 100 g. original straw.

Fungus	Days after inoculation	Loss of D.M.	Total N	Organic N	N factor	N equiv.
<i>T. lignorum</i>	8	39.2	1.26	1.24	0.89	2.2
	16	39.7	1.13	1.11	0.77	1.8
	24	42.6	1.10	1.08	0.73	1.7
	48	51.7	1.12	1.09	0.74	1.4
<i>A. niger</i>	8	41.4	1.35	1.30	0.96	2.3
	16	45.0	1.24	1.18	0.82	1.8
	24	46.0	1.26	1.18	0.83	1.8
	48	48.3	1.20	1.11	0.76	1.5
<i>A. nidulans</i>	8	35.8	1.22	1.20	0.91	2.5
	16	41.2	1.39	1.31	0.84	2.0
	24	43.4	1.14	1.12	0.77	1.7
	48	43.5	1.16	1.13	0.80	1.8
<i>Acre. olivaceospora</i>	8	43.1	1.31	1.23	0.95	2.1
	16	43.2	1.15	1.11	0.78	1.8
	24	45.0	1.13	1.10	0.76	1.7
	48	46.3	1.25	1.23	0.82	1.7
<i>A. terreus</i>	8	37.5	1.34	1.27	0.99	2.6
	16	41.9	1.28	1.25	0.85	2.0
	24	45.4	1.23	1.19	0.84	1.7
	48	51.3	1.20	1.17	0.81	1.6

Series IV (Tables XI and XII).

Stickiness (Table XI). Fungi alone produce no stickiness, but it develops as soon as *S. cytophaga* is inoculated even when the fungus had a start of only a week. This further emphasises the necessity of the presence of fungal tissue for

Table XI. *Decomposition of straw rotted first with fungus for different periods and then inoculated with S. cytophaga and analysed on the 8th and 40th days after inoculation.*

Expressed on 100 g. original straw.

Organism	Days of fungus action	Loss of D.M.	Furfuraldehyde from pentose groups not in cellulose	"True" cellulose	Loss of "true" cellulose	Loss of furfuraldehyde from non-cellulosic constituents	Physical test (g.)
<i>T. lignorum</i>	8	27.8	4.27	23.52	17.4	3.33	3771
	16	36.5	2.93	16.60	24.4	5.67	4452
<i>S. cytophaga</i> for 8 days	24	37.6	3.36	14.10	26.9	5.24	4735
	48	48.5	2.18	9.30	31.7	6.42	4143
<i>T. lignorum</i> +	8	46.2	2.74	10.24	30.7	5.86	5196
	16	47.9	2.32	9.45	31.5	6.28	1998
<i>S. cytophaga</i> for 40 days	24	48.9	1.76	10.03	30.9	6.94	1880
	48	54.9	1.36	7.97	33.0	7.24	2077

the production of stickiness. The stickiness was practically constant at about 4275 g. when *Spirochaeta* acted for only 8 days whereas it varied considerably when it acted for 40 days.

Carbohydrate constituents (Table XI). By comparing the figures in Series I for losses in dry matter with those of *Trichoderma* alone in Table I an estimate can be obtained as to the further decomposition due to *Spirochaeta* in conjunction with the fungus. For instance, the fungus working alone decomposed 16 % of dry matter in 16 days, whereas if working in association with the bacterium for the second 8 days, i.e. a total of 16 days, a decomposition of 27 % was obtained.

Obviously this extra loss must be ascribed to the action of the bacterium. For 48 days with the fungus and 8 days with the bacterium the decomposition amounted to 48 %, which is very close to that obtained with the fungus for 16 days and the bacterium for 40 days. The loss of dry matter for 48 days with the fungus and 40 days with the bacterium is about 55 %, which compares well with 51 % obtained in Series III (b) for a total period of 96 days. The losses in carbohydrate constituents run parallel with the losses in dry matter.

Nitrogen immobilisation (Table XII). The increase in ammonification with the period of incubation explains the drop in the nitrogen factor. *Spirochaeta*

Table XII. *Nitrogen content of straws rotted first with fungus for different periods and then analysed on the 8th and 40th days after inoculation with S. cytophaga.*

Organism	Days of fungus action	Expressed on 100 g. original straw.				
		Loss of D.M.	Total N	Organic N	N factor	N equiv.
<i>T. lignorum</i>	8	27.8	1.49	1.45	1.09	3.9
+	16	36.5	1.36	1.21	0.86	2.3
<i>S. cytophaga</i>	24	37.6	1.32	1.18	0.82	2.2
for 8 days	48	48.5	1.29	1.23	0.89	1.8
<i>T. lignorum</i>	8	46.2	1.33	1.30	0.94	2.0
+	16	47.9	1.38	1.34	0.98	2.0
<i>S. cytophaga</i>	24	48.9	1.30	1.23	0.88	1.8
for 40 days	48	54.9	1.21	1.16	0.81	1.5

has accordingly decomposed the fungus protein for its nitrogen requirements; the excess of ammonia is volatilised, thus accounting for the nitrogen losses.

Series V (Tables XIII and XIV).

Stickiness. The figures for stickiness indicate that the joint action of the two organisms from the very start is unfavourable for its production. The average of 240 g. for stickiness is far below the figures recorded where the fungus had acted alone for at least a week. The low value for stickiness may be accounted for by the failure of the fungus to build up the microbial tissue which appears to be of prime importance in the production of stickiness.

Carbohydrate constituents (Table XIII). The rate of decomposition appears to be very steady, giving a maximum of about 28 % which is approximately that of *Trichoderma* alone for 48 days. In fact, greater decomposition was expected

Table XIII. *Decomposition of straw with fungus and S. cytophaga together for different intervals.*

Organism	Time in days	Loss of D.M.	Expressed on 100 g. original straw.			Loss of furfuraldehyde from non-cellulosic constituents	Physical test (g.)
			Furfuraldehyde from pentose groups not in cellulose	"True" cellulose	Loss of "true" cellulose		
<i>T. lignorum</i>	8	6.9	6.69	37.93	3.0	1.91	139
+	16	11.9	6.52	35.41	5.5	2.08	163
<i>S. cytophaga</i>	24	14.5	5.02	33.77	7.2	3.58	395
together	48	27.9	4.68	20.62	20.3	3.92	266

considering the cellulose-decomposing nature of both the organisms. This low decomposition may partly be accounted for by the apparent failure of the fungus to grow in presence of *Spirochaeta*. The cellulose loss is in proportion to the loss of dry matter. A loss of 13 % dry matter between the 24th and 48th days

corresponds with 13 % loss of cellulose during the same period. The greater part of the loss of organic matter is thus accounted for by the loss in cellulose. There is hardly any increase in the loss of pentose between 24 and 48 days, no doubt due to the inactivity of the fungus.

Table XIV. *Nitrogen content of straws rotted with fungus and S. cytophaga together for different intervals.*

Expressed on 100 g. original straw.						
Organism	Time in days	Loss of D.M.	Total N	Organic N	N factor	N equiv.
<i>T. lignorum</i>	8	6.9	1.37	0.95	0.60	8.6
+	16	11.9	1.28	1.00	0.64	5.4
<i>S. cytophaga</i>	24	14.5	1.04	0.84	0.48	3.3
together	48	27.9	1.30	1.27	0.92	3.3

Nitrogen immobilisation (Table XIV). The organic nitrogen is lower than that obtained with the fungus alone. This may be explained by the inactivity of the fungus. The low nitrogen factor in the early stages may be due to the poor synthesis of fungal tissue in the presence of the bacterium.

DISCUSSION AND CONCLUSIONS.

It appears from these decomposition studies that the production of stickiness in straws depends upon the presence of fungal tissue and the nature of the bacterium. Since *S. cytophaga* has a comparatively low optimum temperature for development, it appears that under the high temperature conditions of fermentation in a manure heap some other organisms must be the main causes of production of stickiness. Stickiness seems to have no correlation with the disappearance of any particular carbohydrate constituent during decomposition.

Although *S. cytophaga* produces gum on a synthetic medium, it fails to produce any stickiness while working upon straws, but in the presence of fungal tissue *S. cytophaga* produces stickiness. It is possible therefore that there is a fundamental difference between the sticky material synthesised by *S. cytophaga* while working upon straw previously decomposed by fungi and the gum it produces on an artificial medium which contains no fungal tissue. Obviously some sticky material other than the bacterial gum is synthesised during decomposition of straw by the interaction between the fungal tissue and the bacterium.

The simultaneous inoculation of fungus and *S. cytophaga* on sterile straw was not very successful either from the point of view of production of stickiness or of general decomposition. The fungus developed with difficulty and two further inoculations were necessary. This may be due either to competition for food material between the fungus and the bacterium or the retarding effect of the end-products of the bacterial action on the growth of the fungus. No counts were made to test this possibility, but it has been observed by Rege [1927] and others that fungi which bring about the initial decomposition of straw eventually disappear and are replaced by bacteria.

SUMMARY.

(1) A number of common soil fungi and two cellulose-decomposing bacteria in pure culture and in different associations have been tested with reference to the production of stickiness and general decomposition.

(2) These fungi and bacteria, while working independently of each other, do not produce stickiness irrespective of the nature of the bacteria.

(3) Fungus decomposition followed by the action of *Mycobacterium agreste* does not produce stickiness.

(4) Fungus decomposition followed by the action of *Spirochaeta cytophaga* produces stickiness.

(5) Progressive decomposition with a fungus and subsequent inoculation with *S. cytophaga* at different stages produce stickiness even if the period of action of the fungus was brief.

(6) Simultaneous inoculations of fungus and *S. cytophaga* produce very little stickiness.

(7) The amount of decomposition effected, the losses in carbohydrate constituents and the nitrogen immobilisation in each case were determined. All the substances studied were removed approximately in proportion to the apparent losses of dry matter.

The author is indebted to Sir John Russell for facilities and to Mr E. H. Richards for suggesting the problem and for invaluable advice and criticism.

The writer's thanks are due to Dr H. Nicol for supplying the pure cultures of bacteria and to Dr Brierley for supplying the pure cultures of fungi. His thanks are also due to Drs A. G. Norman and S. H. Jenkins for their valuable suggestions and criticisms.

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CCXV. THE GLUTEN-DISSOLVING FERMENT OF WHEAT AND BARLEY SEEDS.

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(Received July 10th, 1933.)

In the winter of 1931-32 Miss Kozmin drew our attention to the fact that gluten washed out from wheat grains damaged by the bites of the "tortoise bug" (*Eurygaster* species) within 2 hours at the most following the washing out turns into a liquid. This phenomenon was described by Kozmin [1929; 1930] and Berliner and Koopman [1929], both of whom have suggested that it was probably a case of the action of strong proteolytic ferments. However, testing by the usual method, *i.e.* by autolysis of 5 g. of flour from the affected grain for 6 hours at p_H 4.7 and a temperature of 34° showed only a slight increase of the free amino-groups in comparison with the autolysis of flour from normal grain; the normal grain gave an increase of 2.1 mg. and the damaged grain 2.44 mg. Amino-nitrogen was determined by the indirect method of titration of the carboxyl groups by the Willstätter method, and a difference of 0.34 mg. on a 5 g. aliquot could not be considered decisive. After 48 hours of autolysis there was hardly a change in the difference between normal and damaged flour. The total nitrogen content of the 5 g. of flour being about 120-130 mg. an increase of amino-nitrogen of about 0.3-0.6 mg. certainly could not cause such a liquefaction of the gluten as was mentioned above.

Our next hypothesis was to assume the existence of a specific ferment in the damaged grain dissolving only the proteins of the gluten without splitting the amino-linkages in the polypeptide chains. In order to verify this assumption we have prepared a water extract from the damaged grain by placing in a thermostat a mixture of 1 g. of flour, 5 cc. of water and 1 cc. of toluene, allowing it to stand there for 48 hours at a temperature of $33-35^\circ$ and filtering. From the flour of the undamaged grain fresh gluten was washed off. Aliquots of 1 g. were weighed into Erlenmeyer flasks and to each were added 20 cc. of acetate buffer p_H 4.7, 5 cc. of the ferment extract and 1 cc. of toluene. One part of the flasks was placed for 10 minutes over a water-bath and then in a thermostat for 24 hours. The contents of the second part were analysed immediately and the third part was placed in a thermostat without heating. In the thermostat were also placed flasks containing only gluten without the extract and others containing only the extract without gluten. For analyses the contents of the flasks were filtered through an ashless filter directly into a Kjeldahl flask and carefully washed out. The residue on the filter was digested according to the Kjeldahl method and so was the filtrate mixed with the water used for the washing out. The analyses gave the following results.

	Nitrogen in mg.		
	In filtrate	In residue	Total
Without heating, 24 hours at 33-35°	20.13	50.16	70.29
Heated, 24 hours at 33-35°	15.63	53.56	69.19
Without heating, determined immediately	16.83	53.46	70.29
Gluten alone after 24 hours	6.58	50.37	56.95
Extract alone after 24 hours	13.31	—	13.31
			70.26

After 24 hours' digestion about 8 % of the nitrogen of the gluten passed into the filtrate.

An experiment was undertaken under the same conditions on the effect upon the gluten of a diastatic preparation ("maltin" of Kahlbaum, *viz.* a preparation from sprouted barley). The increase of amino-nitrogen titratable by the Sørensen method amounted after 24 hours only to 1.94 mg. The variation in solubility is expressed by the following values.

	Nitrogen in mg.		
	In filtrate	In residue	Total
Without heating, 24 hours at 33-35°	35.57	26.82	62.39
Without heating, determined immediately	4.28	55.87	60.15
Heated up to 100°, 24 hours at 33-35°	13.64	46.73	60.37
Gluten alone, 24 hours at 33-35°	12.18	44.22	56.40
Diastase alone, 24 hours at 33-35°	3.26	2.83	6.09
			62.49

The results obtained after 6 hours' treatment were also sufficiently definite, *viz.*:

	Nitrogen in mg.		
	In filtrate	In residue	Total
Without heating, 6 hours	16.57	43.50	60.07
Without heating, determined immediately	4.39	56.16	60.55
Heated up to 100°, 6 hours	4.53	55.31	59.84

A still more energetic action was observed in an alkaline medium at p_H 8.0. The results of a corresponding experiment carried on for 24 hours at a temperature of 33-35° with gluten and maltin were the following:

	Nitrogen in mg.		
	In filtrate	In residue	Total
Without heating, 24 hours	47.52	13.66	61.18
Without heating, immediately	5.82	54.49	60.31
Heated to 100°, 24 hours	6.90	55.07	61.97
Gluten alone, 24 hours	4.31	53.70	58.01

The effect upon the gluten at p_H 8.0 of the extract from the grain damaged by the bug gave on the whole the same results as at p_H 4.7.

	Nitrogen in mg.		
	In filtrate	In residue	Total
Without heating, 24 hours	9.16	53.77	62.93
Without heating, immediately	6.47	55.93	62.40
Heated to 100°, 24 hours	6.90	56.22	63.12

The next step was the study of the effect of the dissolving ferment upon the components of the gluten: glutenin and gliadin. The preparations of these proteins were made according to Kiesel *et al.* [1931] from normal wheat flour, p_H 4.7 (acetate buffer). Dry preparations of protein (0.2 g.) were used. The increase of amino-nitrogen titratable by Sørensen's method amounted in the

glutenin for the 24 hours to 3.24 mg. The effect of diastase ("maltin") gave the following results:

		Glutenin				Gliadin				
		Nitrogen in mg.				Nitrogen in mg.				
		In filtrate		In residue	Total	In filtrate		In residue	Total	
Without heating, 24 hours	19.83	19.17	15.03	15.29	34.86	31.21	31.24	5.11	5.33	36.22
	18.48		15.55		34.03	5.55		36.82		
Without heating, immediately	14.53	14.73	20.14	19.29	34.67	20.09	20.66	15.65	15.52	35.74
	14.93		19.45		34.48	15.38		36.79		
Heated to 100°, 24 hours	8.07	7.69	26.45	26.28	34.52	*18.44	19.26	*15.24	14.57	*33.68
	7.30		27.11		34.41	*20.08		*13.90		*33.98
Protein alone, 24 hours	3.21	3.09	28.76	0.68	31.97	19.54	19.31	14.22	15.22	33.76
	2.98		28.60		31.58	19.05		16.22		35.27
Diastase alone, immediately	1.51	1.49	1.22	1.22	2.73	1.51	1.49	1.22	—	2.78
	1.47		1.22		2.69	1.47		1.22		2.69
Diastase alone, 24 hours	1.83	1.83	0.81	0.85	2.64	1.83	1.83	0.81	0.85	2.64
	1.83		0.88		2.71	1.83		0.88		2.71

* Gliadin without the ferment immediately after the experiment was started.

The dissolving effect upon gliadin is considerably greater than upon glutenin.

The enzymic nature of the disintegration of the protein without an increase of the free amino-groups or at least with a considerably smaller accumulation of amino-groups in comparison with the amounts of nitrogen rendered soluble is sufficiently clear from the above experiments. It is hard to imagine what linkages are really being split in the protein molecule during this reaction. It may be believed, however, as is thought by Astbury and Wood [1931], that the protein molecule consists of parallel polypeptide chains connected with each other by means of additional valencies into compound aggregates. Such connections may be formed between 2, 3 or 6 polypeptide chains, which is quite in agreement with the fact found by Svedberg [1931] that the molecular weight of the protein is either equal to 34,500 or 2, 3 or 6 times this. The breaking of the bond between the different polypeptide chains may cause a change of the aggregate state of the protein without freeing new amino-groups. The appearance of the latter is connected with the presence in the ferment preparations of real proteases.

SUMMARY.

1. The gluten of flour from grain damaged by the "tortoise bug" rapidly changes its aggregate condition without any noticeable increase of the free amino-groups.
2. The disintegration of the gluten and of the component parts of it, glutenin and gliadin, which is connected only with a change of the aggregate state of the protein, is a process of an enzymic nature.
3. The corresponding ferment, which is representative of an entirely new type of disaggregating ferments, acts at both acid and alkaline reactions and is found in the seeds of wheat damaged by the "tortoise bug" and in the diastatic preparation from malt.

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CCXVI. SOME LIMITATIONS OF THE CARIUS DIGESTION FOR THE MEASUREMENT OF CHLORIDE IN BIOLOGICAL MATERIAL.

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(Received July 24th, 1933.)

In the quantitative determination of chloride of desiccated biological tissues, particularly those containing fats, it has been amply demonstrated that complete recovery is not obtainable by the method of open Carius digestion with acid. On the other hand the use of preliminary alkaline digestion followed by the open Carius method has been shown by one of us [1933] to give quantitative recovery.

The failure to recover the chloride completely by the Carius method has been interpreted by some, particularly by Morris and Morris [1930; 1932] as due to an actual loss of chloride in some volatile form during the drying process. We have shown that this interpretation is incorrect since we have reported [1931] that if desiccated blood be allowed to stand in contact with water before the Carius digestion, almost complete recovery of chloride results; and more recently one of us has shown [1933] that subjecting dried tissues to preliminary alkaline digestion yields complete recovery as compared with established methods of analysis. The confusion between "diminution in chloride measurement" and an actual loss of chloride persists in the recent article by Norris and Ampt [1933] who have failed to recognise that our experiments indicated not a loss of chloride but rather a failure to measure it.

It was suggested by us that traces of halide may distil off during the digestion of blood and tissues with AgNO_3 and HNO_3 , though in quantities insufficient to account for the diminution in chloride measurement which we reported in the analysis of desiccated biological materials. The following experiments were devised to measure the amount of halide lost during the Carius digestion, using blood as a test material.

Samples of wet and dried blood were digested with AgNO_3 and HNO_3 in a pyrex flask connected by ground glass joints to the digestion flask of a Thompson-Oakdale apparatus¹. The fumes given off from the digestion mixture were passed through hot concentrated H_2SO_4 in the digestion flask of the Thompson-Oakdale apparatus, and after having passed through quartz tubing maintained electrically at cherry-red heat they were collected in a solution of Na_2HAsO_3 . The residual fumes following the Carius digestion were drawn through the Thompson-Oakdale

¹ In some of the experiments a similarly modified Willard-Thompson apparatus was employed.

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apparatus in a stream of chloride-free air. The Na_2HAsO_3 solution was neutralised with HNO_3 , and the halide precipitated with AgNO_3 . The results of the analyses are given in Table I. The estimations of the halide recovered from the fumes were made by comparing the turbidity of the unknown with that of freshly prepared suspensions of AgCl . By comparison of turbidity we were able to estimate any chloride that might be lost up to 3 mM per litre of blood within $\pm 0.1 \text{ mM}$.

Table I.

Material	Remarks	Maximum halide re- covered from fumes from Carius digestion.	Chloride analyses.
		Milli-equiv./l. wet sample	Milli-equiv./l. wet sample
Ox blood 16	Wet samples dried in flasks at 110° for 24 hours	0.2	80.1
		0.5	66.8
Ox blood 18	Wet samples dried and subjected to preliminary alkaline digestion	0.3	—
Human blood	(a) Wet samples	0.5	—
	(b) Dried and finely pulverised samples	1.0	—
	(c) Samples dried in flasks	1.8	—
NaCl sol. 100 milli-equiv./l.	—	0.1 (same as reagent blank)	—
Human blood	(a) Wet samples	—	83.2
	(b) Dried samples subjected to preliminary alkaline digestion	—	83.8
	(c) Wet samples digested in flasks connected with stoppered re- flux condensers	—	83.0
	(d) Dried samples digested in flasks connected with stop- pered reflux condensers	—	68.8

It will be seen from Table I that the minute amount of halide which we were able to recover from the fumes during the Carius digestion of blood accounts only to a very small extent for the failure to measure chloride completely by the direct Carius method with dried blood. Moreover, when dried blood was subjected to direct Carius digestion in Erlenmeyer flasks connected by ground glass joints to stoppered reflux condensers, failure to measure chloride completely was still experienced. These studies suggest that failure to measure the chloride completely in desiccated biological tissues must be attributed to other factors rather than to a loss of chloride.

We are in agreement with Norris and Ampt as to the desirability of alkaline digestion of certain biological materials before proceeding with the measurement of chloride. In the case of wet blood or serum, however, preliminary alkaline digestion was found unnecessary.

Our studies demonstrate that in some biological materials chloride is not completely measured by the direct open Carius method. Preliminary leaching with water in some instances provides almost complete recovery of the chloride; preliminary digestion with alkali is completely effective and is, therefore, the

procedure of choice in dealing with such materials. We have been unable to demonstrate that this failure to measure chloride by the direct open Carius method is due to a loss of chloride in amounts greater than 1.8 *mM* per litre of blood.

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CCXVII. ON MONOLAYERS OF PROTEOLYTIC ENZYMES AND PROTEINS.

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(Received August 25th, 1933.)

PART I.

THE REACTION KINETICS.

IN a recent communication [Hughes and Rideal, 1933] it was shown that the progress of reactions taking place at an air-liquid interface could be followed by observation of the rate of change of the phase boundary potential. In this paper an account is given of a preliminary investigation of the action of a typical proteolytic enzyme system, trypsin on monolayers of egg-albumin and of caseinogen. This investigation was undertaken because it appeared probable that this type of reaction at a phase boundary frequently occurs in living systems.

The structure of albumin monolayers.

There is a considerable amount of information which indicates that the structure of proteins in bulk solution is different from that which exists when the protein is extended as a monolayer in a film.

The measurements of Svedberg and Nichols [1926] lead us to regard egg-albumin as an isodisperse protein of molecular weight 34,500, and from the constancy of its "molecular" weight it is assumed to be approximately spherical in form [*cf.* Sørensen, 1915]. The spherical character of the egg-albumin molecule is further supported by an optical examination due to Ettisch *et al.* [1931] who found that egg-albumin solutions in flow exhibit no birefringence and light scattered in the Tyndal beam is polarised. These views are opposed to those of Staudinger who regards eucolloids as existing in the filamentous or distended state in solution. The ease with which the protein particle breaks down outside the region of its stability limits, p_H 4 to p_H 9, suggests that the particle may not be really isodisperse within the limits but may consist of three units each of true molecular weight 11,500, built up into a single net-like unit (the choice of three similar units is based upon the approximate analysis by Vickery and Shore [1932]). A net-like structure has indeed been proposed by Kuhn [1932] for gelatin from an analysis of the viscosities of dilute solutions of this protein. It is clear that whichever view of the structure of egg-albumin in solution be correct, the balance of evidence is against the rod-like or filamentous structure. It has been shown [Hughes *et al.*, 1932; Fosbinder and Lessig, 1933] that a number of proteins may be spread directly as homogeneous surface monolayers from the solid material. Protein monolayers are characterised by the fact that under suitable conditions they form highly elastic gel-like films. For the polydisperse proteins such as gliadin and caseinogen in which the polypeptide chains are probably highly branched the gel state is not acquired until relatively high two-

dimensional pressures (15 dynes/cm.) are applied to the film; this phenomenon is accentuated in films of polypeptide mixtures such as peptone where solid films are formed only under very high compression. In the case of the isodisperse proteins like albumin the characteristic gel-like appearance is quite noticeable at relatively low pressures (< 1 dyne per cm.) and even below these pressures the films cannot be regarded as anything except weak gels developing into more homogeneous gels. If egg-albumin be spread on buffer solution it is found that the rate of spreading and the quantity per cm.² attained at equilibrium suddenly increase several 100 % when the acidity of the substrate is extended beyond the stability region of the isodisperse state; it may be significant that this change coincides with the region of peptic digestion of the film.

Monolayers of the isodisperse and polydisperse proteins are also differentiated by the fact that in the polydisperse proteins such as gliadin and caseinogen, possessing many branched chains, the phase boundary potentials are relatively high whilst for the monodisperse proteins such as albumin they are much lower. The examination of the effect of changing the p_H of the substrate on the phase boundary potential likewise indicates that this is governed by the number and nature of the polar groups in the side-chains and is scarcely affected by the less polar amide linkages in the main polypeptide chain.

In the solid state Astbury and Wood's analysis [1932] has shown that the proteins exist in two characteristic forms—the zig-zag type of extended polypeptide chain, the β -keratin structure, and a chain folded in a series of hexagons of periodicity 5.1 \AA. , the α -keratin or diketopiperazine ring type. Whilst in the surface soluble proteins spreading out into monolayers the chains must be of the original β -keratin type, it seems certain that conversion into the α -keratin or diketopiperazine ring structure takes place in the surface and very rapidly, and it is to this latter structure that the stability of the films is due. Whilst the measurements of the limiting surface concentrations at the stage when gel formation commences are not sufficiently well defined experimentally to confirm this view as to their structure, yet the values obtained for the isodisperse proteins where the branch chains are not so prominent certainly favour this hypothesis. The following experimental observations may be cited in support of this view. The decrease in solubility of natural proteins after being converted into a film is well known and has been examined in detail by many investigators, notably Ramsden. Monolayers of egg-albumin may be heated to 85° and quickly cooled without apparent alteration in their physical properties. Natural albumin is readily dispersed by caustic soda more concentrated than $p_H 11$ but monolayers of albumin are very resistant even to $N/10$ caustic soda. On allowing even dilute solutions of proteins to stand [cf. du Noüy, 1926] the micelle in solution comes up to the surface and there spreads, undergoing the process of denaturation and loss of solubility. Finally, albumin in the film state is readily attacked by enzymes, a property not associated with natural albumin in bulk which appears to be very resistant, and this ease of attack is associated with the presence of diketopiperazine and anhydride rings in the molecule [Matsui, 1933]. It might be suggested that the process of denaturation at interfaces is associated with or may even consist in the conversion of the β -keratin or zig-zag structure into the α -keratin or diketopiperazine ring form.

We have noted that the examination of the phase boundary potential of a number of protein monolayers suggested that the main contribution to the phase boundary potential was from the polar groups in the side-chains and that the $-\text{CO}-\text{NH}-$ linkage of the main chain contributed but little. On hydrolysis, if only one or more of these links be broken to form shorter polypeptide

chains terminating in $-\text{COOH}$ and $-\text{NH}_2$ respectively, the phase boundary potential should increase until the peptide chain becomes so small that dissolution commences, and only then will the phase boundary potential commence to fall. As an alternative method, hydrolysis consists in the breaking either of an amide linkage near the end of a chain or two amide linkages close together in a chain and the small fragment containing relatively many polar groups thus released goes into solution. Under these conditions there will be a gradual fall in the phase boundary potential as the protein passes through the stages of peptone, polypeptide, tri- and di-peptide and simple amino-acid disintegration. As will be noted, this latter change is the one found experimentally and it is indeed possible to express, in a qualitative manner at least, the stages of disintegration of the protein by observation of the magnitude of the phase boundary potential.

Since the concentration necessary to form films is of the order of 10^{-7} g. per cm^2 , the method of surface potentials is not only particularly well suited for the characterisation and control of purity of biological materials which possess large surface potentials and are available only in small quantities but also in its sensitivity in measurement of chemical reactivity and rates of reaction for substances in almost sub-analytical quantities. In the case of these enzyme reactions in particular the kinetics can be followed over a convenient period of some twenty minutes at room temperature, and, furthermore, some light is shed on the actual nature of the enzymes themselves.

Thanks to the investigations of Willstätter and Grassman, Waldschmidt-Leitz and his co-workers and Northrop¹, we can distinguish in commercial trypsin solutions digesting egg-albumin at p_{H} 8-9, a proteinase, tryptase, effecting a primary hydrolysis into polypeptides; further hydrolysis proceeds by attack at the terminal groups in the polypeptide chain by polypeptidases. We may note that both the amino- and carboxy-polypeptidases which may be present in trypsin hydrolyse the amide linkages close to a terminal $-\text{NH}_2$ or $-\text{COOH}$ group respectively with the result that small soluble fragments, *e.g.* leucine or tyrosine, are removed from the ends of the polypeptide chains, a point, as we have noted, of some importance in interpreting the effect of hydrolysis on the phase boundary potential. Whether a small soluble fragment is liberated in the action of the primary proteinase, tryptase, on the original protein has not been established but, as we shall note, such a suggestion is supported by the observed change in phase boundary potential. Still further hydrolysis is effected by the dipeptidases present.

EXPERIMENTAL PROCEDURE.

The experimental method employed followed closely that described in previous communications [Schulman and Rideal, 1931; Schulman and Hughes, 1932], with the exception that the apparatus was kept at 25° , the egg-albumin being spread from the solid from a micro-balance on the surface of $M/20$ buffer solutions made up according to the directions of Clark ("Determination of hydrogen ions") over a range of p_{H} 2-12; the solution was contained in a quartz trough of 300 cc. capacity. It was found necessary to take precautions to eliminate the formation of diffusion potentials in the liquid junctions connecting the buffer solution in the trough with the auxiliary electrode and membranes on the electrode itself.

The protein film so spread could be compressed by means of glass barriers to

¹ Compare *Ergebnisse der Enzymforschung*, Band 1, Leipzig, 1932.

the stage where the phase boundary potential on gelation no longer varied appreciably on further contraction; the film in this state possesses a characteristic surface potential dependent only on the p_H of the substrate. The water surface behind the barrier which had been moved to compress the film was left clean. 20 cc. of a suitable solution of the enzyme having stood at room temperature for half an hour in the same buffer solution as that on which the protein film was spread, but $M/10$ in strength (strong buffers being found necessary owing to the acidity of the trypsin), were injected and well mixed from the free side of the barrier into the underlying solution. With the aid of the moving polonium electrode the process of subsequent digestion of the protein film as well as the rate of adsorption of the ferment at the clean surface could be followed.

Owing to the high adsorbability of albumin at the surface of aqueous solutions of albumin, one part in half a million parts forming solid gel films at an air-liquid interface, it was found essential to eliminate soluble proteins from the enzyme. If the soluble protein is not eliminated it forms partially digested protein films at the surface which obscure and inhibit the main reaction so that the reaction appears to proceed in steps or jumps, the dimensions of which are dependent on the time the enzyme has been in solution before being injected into the trough. It was found that on standing at room temperature for half an hour the minute quantities remaining from a Merck's pancreatin were removed presumably by autodigestion. Solutions of pancreatin prepared in this manner were found to possess definite and reproducible phase boundary potentials, which could be employed as a criterion for their freedom from protein. All experiments were carried out with Merck's pancreatin, which gave more uniform results than other varieties.

The change in phase boundary potential due to an albumin film in the gel state on the buffer solution at p_H 7.7 is $\Delta V = 230$ mv. On injection of the pancreatin solution a steady decrease in the phase boundary potential is observed and the film loses its rigidity when the surface potential attains a value of *ca.* $\Delta V = 180$ mv., finally becoming quite fluid. The phase boundary potential finally attains a constant value of $\Delta V = 118$ mv. This value $\Delta V = 118$ mv. is identical with the characteristic value shown by a solution of pancreatin at this p_H so it appears that the protein film has been completely digested. On the protein-free side of the barrier within two minutes of injection of the trypsin solution a potential change of $\Delta V = 118$ mv. is obtained; the capillary active constituents in the pancreatin thus form a vaporous film of characteristic surface potential whose value does not change even on relatively rapid compression or expansion of the surface, a behaviour similar to that of substances which are capillary active and on solution in water form a Gibbs layer which may be regarded as a two-dimensional vapour or gas.

During the reaction the original protein gradually disappears from the film and we may assume that the removal of fragments of the protein from the surface is reflected in the change of phase boundary potential; this assumption is borne out by the fact that identical velocities of reaction are obtained when, instead of measuring the rate of change of phase boundary potential with time, maintaining the area of film constant, the rate of contraction of area of film necessary to maintain a constant phase boundary potential is measured. Experimentally, however, this latter procedure is difficult to carry out, especially in the later stages of digestion.

In Fig. 1 are shown the effects of variations in the concentration of pancreatin and of temperature on the rate of digestion of an albumin film at p_H 7.7.

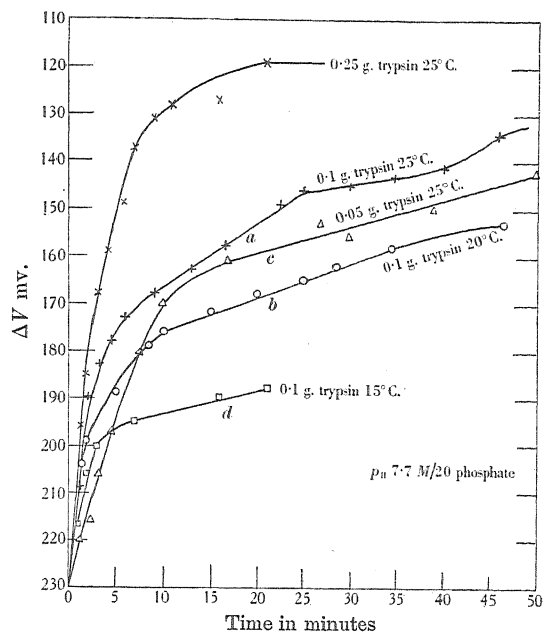


Fig. 1.

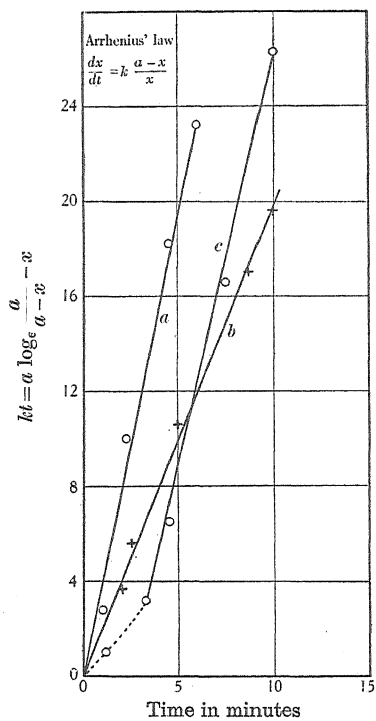


Fig. 2.

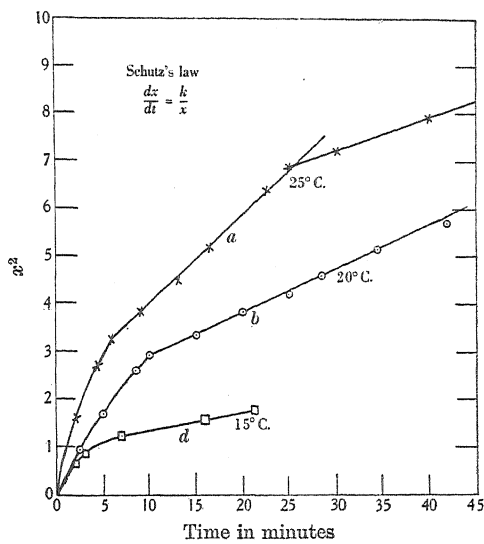


Fig. 2a.

It will be noted from Figs. 2 and 2a that the reaction in presence of pancreatin, originally unimolecular, becomes converted on decreasing the concentration of pancreatin and at lower temperatures firstly into an inhibited reaction obeying the Arrhenius expression $\frac{dx}{dt} = k \frac{a-x}{x}$ (Fig. 2), then the rate of displacement of the by-products of the digestion by the trypsin alone governs the reaction velocity and the reaction follows Schutz's law $\frac{dx}{dt} = \frac{k}{x}$ (Fig. 2a). Curves *a*, *b*, *c* and *d* are given as examples for the two expressions, *c* showing irregularity owing to diffusion to the surface of a weak trypsin solution.

A comparison of the actual velocity constants obtained for the digestion of films of caseinogen with the approximate values obtained for the reaction in bulk phase under similar conditions indicates that they are of the same order of magnitude, the chief difference is that the transition from a unimolecular surface reaction velocity to an inhibited reaction following Schutz's law takes place as a result of a smaller change in the concentration of the pancreatin; this is doubtless due to the greater facility with which by-products from the reaction can diffuse away from the disperse micelle in the bulk phase and the decreasing ability of the trypsin in the lower concentration to displace the by-products formed initially on the surface, since on complete digestion the pancreatin ultimately displaces all the by-products of digestion, as indicated by the attainment of identical values of ΔV_x on both sides of the barrier.

In Fig. 3 is plotted the logarithm of the change in phase boundary potential with the time and in Fig. 4 the unimolecular velocity constants respectively as a function of the p_H over the range of activity of the pancreatin. It will be noted that the surface reaction follows very closely that of the bulk reaction revealing a marked optimum between p_H 8 and p_H 8.4.

In the neighbourhood of the optimum the reaction only reveals a unimolecular reaction constant over the first few minutes, by-products being formed so rapidly that inhibition of the reaction sets in after a short period; thus the reaction as a whole proceeds more rapidly at p_H 7.5 than at p_H 8.2.

It is somewhat remarkable that the reaction at p_H 7.5, in spite of the complexity of the process, appears to be uniformly unimolecular over the whole course of the reaction, suggesting that the slowest of the enzyme reactions is that involving the first breakdown by the proteinase. Another noticeable feature of these reactions in films is that occasionally (approximately one experiment in every twenty) a period of induction was noted before the reaction commenced with its normal velocity, the usual phenomenon met with in the tryptic digestion of albumin when studied in bulk phase.

The specific proteolytic enzymes.

In order to examine the mechanism of tryptic hydrolysis in more detail, a number of experiments were carried out with proteinase and carboxypolypeptidase. We are indebted to Prof. J. H. Northrop of the Rockefeller Institute at Princeton for a sample of proteinase or crystalline trypsin (N) prepared by ammonium sulphate precipitation from pancreatin and also to D. R. P. Murray of the Sir William Dunn Biochemical Laboratory at Cambridge for a sample of proteinase (M) and of carboxypolypeptidase (P) prepared according to the method of Waldschmidt-Leitz by preferential adsorption on alumina.

In contrast with solutions of pancreatin, which at p_H 7.7 form only vaporous films with very low phase boundary potentials, these specific proteolytic ferments form solid gel-like films, possessing characteristics very similar to films of egg-

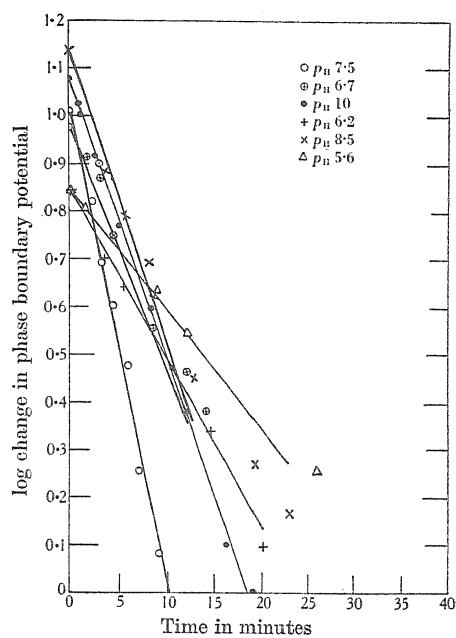


Fig. 3.

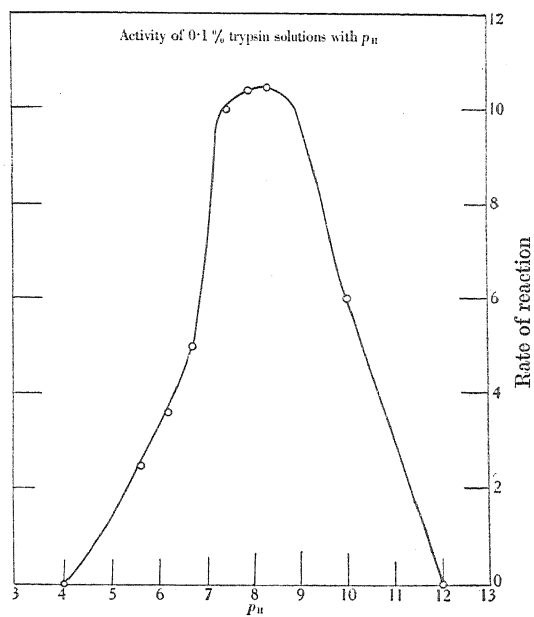


Fig. 4.

albumin, the phase boundary potential of the proteinases being $\Delta V = 220$ mv. and of the carboxypolypeptidases $\Delta V = 200$ mv. The proteinase N formed solid films very rapidly after injection of the solution into the buffer solution in the trough but some ten and twenty minutes respectively elapsed before the proteinase M and the carboxypolypeptidase P yielded solid films.

Solutions of these proteases were injected at p_H 7.7 under films both of egg-albumin and of caseinogen. In the examination of the stages of proteolytic digestion in films, caseinogen being polydisperse possesses certain advantages over egg-albumin in that not only is the change in phase boundary potential greater than for albumin in the region of gelation, but, accompanying a high surface pressure the region of gelation is smaller and more sensitive to changes in pressure.

In Figs. 5 and 6 are given a summary of the experimental results of the action of these enzymes on the two proteins.

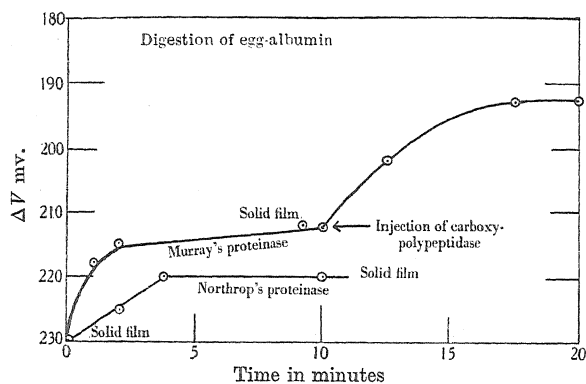


Fig. 5.

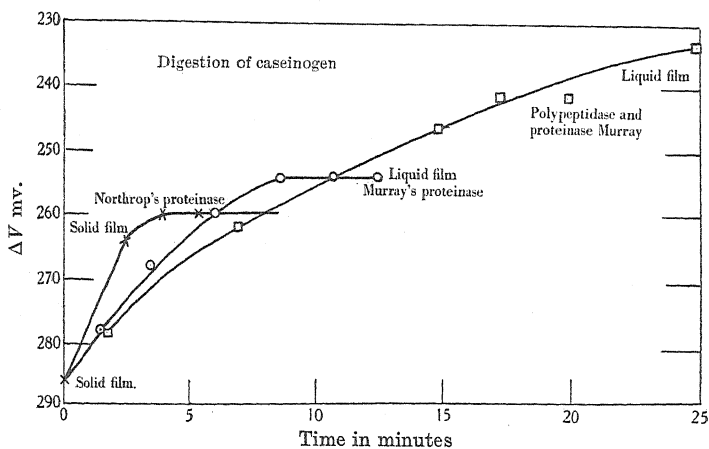


Fig. 6.

It will be observed that the proteinases N and M reduce the surface potential to a definite value, M, to a slightly greater extent than N, a fact probably due to the difficulty in elimination of the last traces of polypeptidases by the adsorption

process. Owing to the fact that only small quantities were available it has been impossible to determine the reaction constants, but, with the exception of the digestion of egg-albumin by Northrop's proteinase which follows a zero order, they appear to be unimolecular. In one case the carboxypolypeptidase P was injected into the solution of proteinase which had already digested a film of egg-albumin to the first stage. This resulted in a further stage of digestion with a concomitant fall in the surface potential of a further 20 mv. Likewise a mixture of M and P enzymes digested a caseinogen film with a change of 55 mv. compared with 25 mv. with the proteinase alone.

On comparison of these values with those obtained in the digestion of either egg-albumin or of caseinogen by pancreatin containing the complete series of enzymes, it is found that in the proteolytic digestion by the proteinase N about 10 % of the original protein has left the surface in some soluble form, whilst a further 25 % leaves the surface through the addition of the carboxypolypeptidase P.

On the other hand, about 15 % and a further 16 % leave the surface in the action of the ferments N and P respectively in the case of caseinogen. Another interesting feature observed in the digestion of a film of caseinogen, which possesses a region of gelation very sensitive to pressure (the rigidity of the gel being imperceptible when the phase boundary potential falls below 260 mv.), is that it is still in a gelatinous state when digested by the proteinase N, but on digestion by the ferment M which contained a slight trace of a carboxypolypeptidase a small further digestion reflected by a change of only 4 mv. in the phase boundary potential caused it to assume a fluid condition. The proteinase, however, rising to the free surface of the water on the other side of the glass barrier, forms a solid film. These conditions on each side could be maintained for several hours. The proteinase which itself forms a solid film does not displace the liquid film of the partly digested caseinogen owing to the higher surface pressure of the latter, and continuous gel-like films of proteins do not appear to be formed except at an air-liquid interface, no formation taking place underneath a monolayer.

PART II.

SOME STRUCTURAL CHARACTERISTICS OF THE ENZYMES.

The phase boundary potential of trypsin.

In Fig. 7 are plotted the phase boundary potentials of an albumin film in the gel state with that of a solution of trypsin for comparison as a function of the p_H of the medium.

It is interesting to note that at p_H 4 trypsin causes a marked alteration in the phase boundary potential, viz. $\Delta V = 320$ mv.; no tryptic activity is noted here, the film is gel-like and possesses all the properties of an albumin protein film. As we pass into the region of proteolytic activity the phase boundary potential falls, the surface loses its gel-like character and becomes perfectly fluid and similar to that of a vaporous or gaseous two-dimensional phase. Pepsin exhibits a similar behaviour, forming an albumin-like film at p_H 7 but a fairly mobile surface with a low phase boundary potential at p_H 2, the peptic optimum. The experimental evidence suggests that the trypsin solutions, even when prepared with the precautions stated, still contain small quantities of protein which form films at p_H 4; but at p_H 8 the rate of arrival of the protein micelle from the interior with

the subsequent spreading is equalled by the rate of tryptic digestion of the protein. In the region p_H 8 to 12 the phase boundary potentials are very similar as well as the fluidities of the surface, but at p_H 12 no surface tryptic activity is noted. It is probable that the traces of protein left in the solution of the enzyme in the micellar form are completely dispersed at this p_H and thus cannot form surface films by spreading from micelles. With strong solutions of trypsin or on

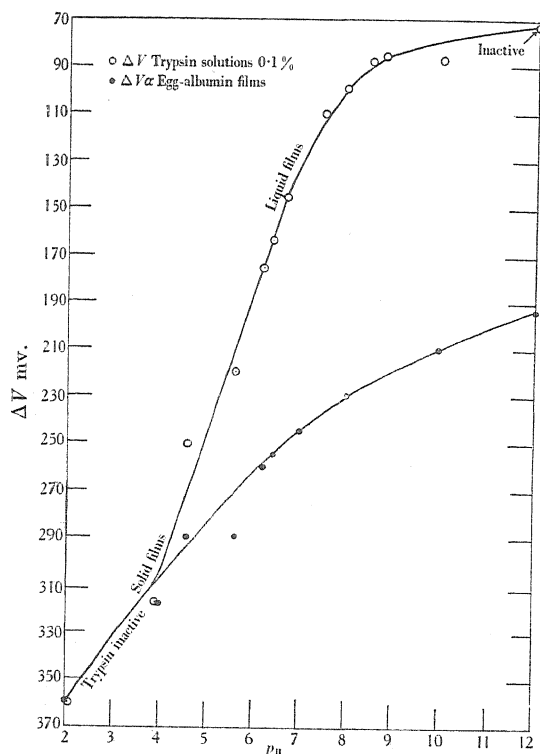


Fig. 7.

the addition of extra albumin solid gel-like films are eventually formed. Albumin when spread from a solid on to the surface forms a perfectly stable gel-like film at this p_H . It is clear that the characteristic surface potential which remains almost constant over this range of p_H , whilst no criterion for the activity of the ferment, is an indication that a distinct change has taken place in respect to the protein constituents in the surface solution.

That the films formed by pancreatin on acid substrates really consist of protein-like material was confirmed by the fact that they are readily digested by pepsin injected into the solution at p_H 2 in the usual manner.

The resultant active surface of pancreatin at the optimum p_H evidently contains no protein, although at p_H 2 undigested pancreatin forms films with all the characteristics of protein.

Similar digestion experiments were carried out with pepsin which forms protein-like films on alkaline solutions (p_H 7.7) of slightly lower $\Delta V = 220$ mv. compared with 240 mv. for albumin. These could be digested readily by pan-

creatin. Likewise the protein-like films formed by the proteinases obtained from Prof. Northrop and Dr Murray (see Part I) as well as by the carboxypolypeptidases could be digested in a similar manner by further injection of pancreatin.

Since the protein-like proteinase and the more disperse protein-like carboxypolypeptidase are further digested by addition of the lower trypsin, it would suggest that the various tryptic ferments are associated with their own breakdown product of original protein when in solution at the p_H for optimum activity, and over the inactive region of p_H with proteins albumin-like in character.

It is clear that all these enzyme preparations are associated with the presence of protein-like material which is capable of being digested by other enzymes or by autodigestion and, also, after removal of the protein by digestion it appears that the surface enzymic activity of the solutions remains unimpaired.

The conclusions inferred from these experiments could logically be extended to enzyme solutions which have been digested by other enzymes, *e.g.* pancreatin by pepsin. These have already been examined by Northrop who showed that the enzymic activity in solution decreased as the digestion of the proteinase proceeded. We have found by digesting pancreatin with pepsin at p_H 2 and then bringing the solution back to p_H 7.7 that in agreement with Northrop's observation the tryptic activity in bulk disappears as the peptic digestion becomes more complete. On the other hand the very interesting observation was made that whereas these solutions were inactive for the digestion of caseinogen, albumin or gelatin in the bulk phase, if films were formed of either of the former proteins on these apparently inert solutions, digestion took place rapidly with unimpaired activity.

The method consisted in dissolving 0.3 g. pancreatin and 0.2 g. pepsin in 50 cc. *N*/100 HCl, leaving to digest for varying lengths of time at 38° and injecting the mixture into the trough containing 250 cc. phosphate buffer at p_H 7.4. The changes in films of egg-albumin or caseinogen spread upon the surface were then observed. It was noticeable that on prolonged digestion of the pancreatin by pepsin the surface proteolytic activity became greater until it reached the normal activity of undigested pancreatin after 24 hours' treatment. This was probably due to the removal from the solution by peptic digestion of protein which inhibits the surface reaction. Even after 48 hours' digestion by pepsin a considerable surface tryptic activity was observed. A control experiment indicated that the tryptic activity of pancreatin appeared to be unimpaired when it was dissolved in 50 cc. *N*/100 HCl and the solution subsequently injected into a phosphate buffer at p_H 7.4.

In the curves in Fig. 8 are shown the results obtained and it is seen that this activity for surface digestion is maintained for relatively long periods of time; whilst after only 5 hours' digestion of pancreatin by pepsin the activity in the bulk phase is scarcely measurable when tested with caseinogen and precipitation by acetic acid. Pepsin, on the other hand, is quite inactive on protein films at p_H 7.7 and when the acidity is brought back to p_H 2 it is still completely inactive; this is analogous to its behaviour in the bulk phase.

From the curves in Fig. 1 (Part I) it is seen that the surface activity is very sensitive to changes in the bulk concentration of pancreatin, and we must conclude that in these experiments the concentration of active enzymes in the bulk phase has been left unaltered by the peptic digestion, although the solutions exhibit no tryptic activity in the bulk phase.

Two points emerge from these film experiments; firstly, that the active enzyme can be removed from its protein-like carrier by suitable digestion. This appears to be in agreement with the experiment of Dyckerhoff and Tewes [1933],

who effected an exchange of protein carrier for the pepsin, making edestin and other protein carriers peptically active by precipitation from pepsin solutions, whilst Frenkel [1932] effected a separation of a toxin from its protein carrier, leaving an active solution containing no protein in the case of diphtheria toxin.

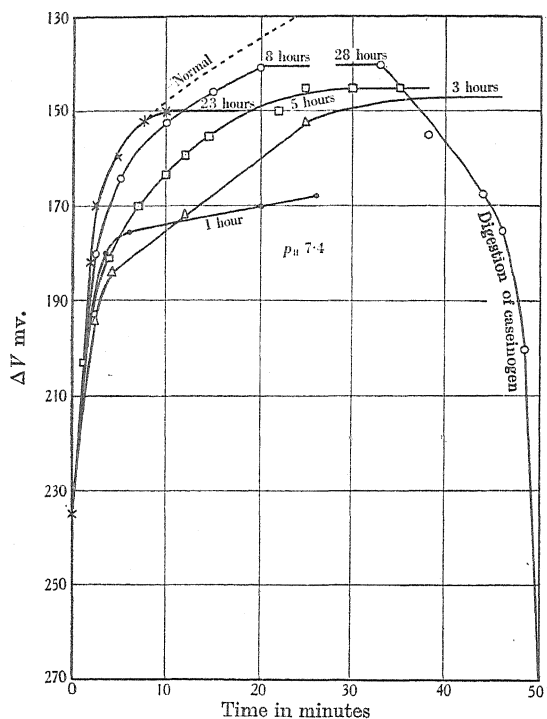


Fig. 8. Digestion of albumin films by trypsin solutions which have previously been digested by pepsin at p_H 2. 0.2 g. pepsin + 0.3 g. trypsin in 50 cc. HCl; in bulk, no reaction after 5 hours.

The second point of interest is that solutions of these enzymes from which their normal protein-like carriers have been removed by digestion are incapable of exerting any enzymic activity on proteins except when these latter are presented to them in the particular state or orientation associated with the film. We have so far been unsuccessful in effecting a regeneration of bulk enzyme activity by the addition of a suitable support material, although we have included both inactive enterokinase and edestin, as well as albumin denatured in various ways, including that denatured by film formation, and also substances such as alumina and quartz. The experimental investigation is being continued in this direction.

It has long been known that destruction of enzymic activity in respect to its activity in a bulk phase proceeds rapidly on boiling. A series of experiments was carried out with a view to investigating the existence (in boiled solutions) of any residual enzyme activity to protein monolayers. Pancreatin solutions containing 0.3 g. in 20 cc. of buffer solution were boiled for various periods of time and cooled rapidly to 0° and then injected into the trough containing the buffer solutions and their surface enzymic activity measured by the rate of hydrolysis of either albumin or caseinogen films; comparative runs with un-boiled solutions were carried out at the same time. The results obtained are shown in Figs. 9 and 10.

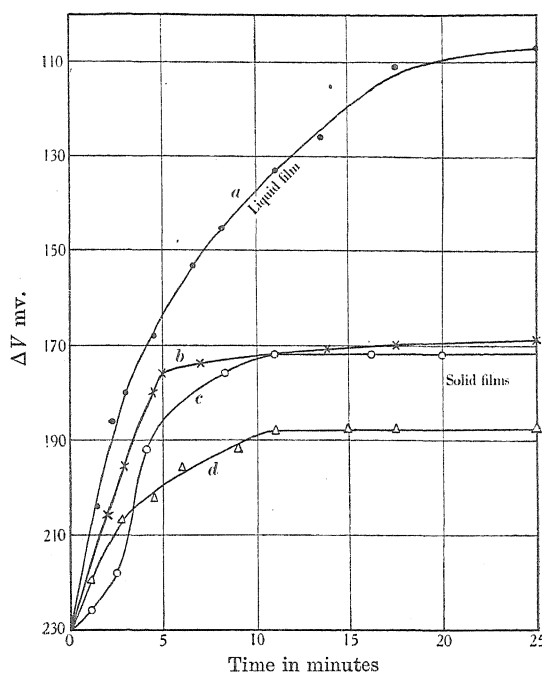


Fig. 9. Digestion of egg-albumin by boiled trypsin solutions p_H 7.8. *a*. Normal 0.1% trypsin solution. *b*. Solution heated for 5 minutes. *c*. Solution heated for 30 minutes. *d*. Solution just brought to boiling.

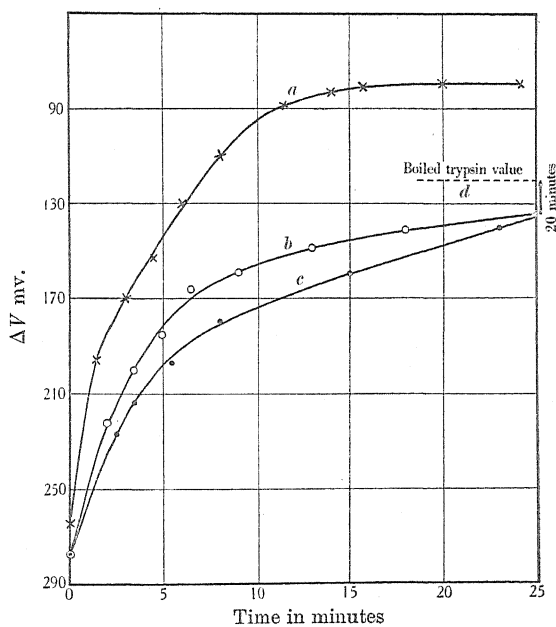


Fig. 10. Digestion of caseinogen by boiled trypsin solutions p_H 8.2. *a*. Normal. *b*. Heated to boiling at p_H 8.2. *c*. Boiled for 30 minutes at p_H 8.2. *d*. Boiled for 30 minutes at p_H 4.6 and filtered, no reaction.

It will be observed that solutions of trypsin when boiled at p_H 8 for a period even as long as 30 minutes still exert a marked tryptic activity on protein films, the activity being still about one-third that of the original solution, whilst no activity could be noticed in bulk as tested by caseinogen and acetic acid precipitation. It may be remarked that egg-albumin films appear to be more resistant to the boiled enzyme than films of caseinogen, which liquefy and disappear in the normal manner. The ultimate phase boundary potentials obtained are some 40 mv. higher for the boiled than for the unboiled solutions, showing that some change has occurred in the component parts of the pancreatin.

The results of these experiments carried out at p_H 8 are to be contrasted with the results obtained by boiling a trypsin solution at p_H 4.6, filtering off the coagulated protein and bringing the p_H back to 8 again. These solutions exhibit no tryptic activity either in bulk or for protein films and indeed form solid protein-like films themselves at an air-liquid interface which, however, in no way affect the solidity of a gelatinous caseinogen film if a solution of the boiled enzyme be injected underneath. It appears that the enzyme surface activity is in direct relation to the degree of coagulation and consequent insolubility of the protein carrier; for at p_H 8 there is only a slight precipitation of coagulated protein and a lengthening of the digestion period of a film from some fifteen to forty-five minutes.

It might be concluded from these experiments that the enzyme consists of at least two components, a "free enzyme" non-protein in character and a protein possessing a specific structure, generally adsorbing or reversibly binding the free enzyme. The "free enzyme" readily reacts with proteins when presented to it in the form of an orientated protein monolayer but does not react with proteins in solution. To impart activity for a bulk phase reaction to the "free ferment" the latter must be adsorbed on a protein possessing a specific structure, for although it is readily adsorbable by coagulated proteins it is inactive both for surface and bulk reaction in this state. It is possible that the function of active enterokinase is to present such a surface.

It may be supposed that an enzyme consists of a system which includes several separate components, the "free enzyme," which in the case of the proteolytic enzymes appears to be non-protein in character, the free enzyme carrier which is specific and protein-like in character and behaves in a manner similar to that of an orientated monolayer and of surface activators such as enterokinase. The activity of the complex of free enzyme and its carrier is dependent on its environment both in respect to its power of adsorption and its chemical reactivity. It is hoped that further experiments on the relationship between bulk and surface reactivity will throw further light on the characteristics of the enzyme system.

It is interesting to note here that when these proteolytic ferments are treated chemically, *i.e.* with acids or alkalis or by poisoning with metallic ions such as Ag^+ or Cu^{++} , their activity is destroyed equally both for bulk and surface reactions; on the other hand, when they are treated by boiling or the protein removed by digestion by another enzyme, only the bulk activity is impaired.

The interpretation advanced for these experiments revealing the peculiar relationship existing between the activity of what has been designated the "free enzyme" and proteins when presented to the "free enzyme" in the form of an orientated monolayer is based upon the hypothesis that the changes in phase boundary potential observed when a solution of an enzyme is injected under the surface of a buffer solution covered with a protein monolayer can be taken as a measure of enzymic activity, *i.e.* a digestion of protein by the enzyme. In

support of this hypothesis may be advanced the results of the investigations described in Part I where the process of gradual liquefaction of a protein film by an enzyme was described, the coincidence of the p_H optima for both bulk and surface reactions, the fractional changes effected by the proteinase and carboxypolypeptidase respectively, and lastly the various forms of what have been termed velocity curves obtained with different concentrations of enzyme, which curves are faithful replicas of the velocity curves obtaining for bulk solution in these systems.

It seemed desirable to show more directly that the disappearance of a protein film from the surface of a buffer solution after injection into that solution of a proteolytic enzyme was in reality a digestion of the protein.

A solution containing both egg-albumin and pancreatin at p_H 8 was allowed to undergo digestion. From time to time a few drops of the solution were placed by means of a dropping pipette on the surface of a buffer solution at p_H 8 and the change in phase boundary potential noted. The phase boundary potential in all cases attained the characteristic value obtained for the original protein with the formation of a solid film until complete digestion had taken place as determined by the absence of a precipitate with acetic acid, when the value characteristic of solutions of trypsin would appear and no solid film be formed. Evidently undigested protein in the drops spreads on the surface in preference to the trypsin or the products of hydrolysis until no protein is left in the solution. The identity of the phase boundary potential with that of the protein reveals no evidence of a protein-trypsin complex, although, as will be noted in the next series of experiments, evidence for the existence of a complex between some constituent in the pancreatin and a breakdown product of egg-albumin can be presented.

In the next series the trough was filled with buffer solution containing pancreatin and egg-albumin in various concentration ratios and the phase boundary potential determined from time to time as the process of bulk digestion proceeded.

It was found that the phase boundary potentials shown by these solutions were very sensitive to the ratio of the pancreatin to albumin. When the original solution contains a slight excess of pancreatin to protein the surface of the solution is found to acquire a solid film rapidly with all the characteristics of the protein, but this liquefies in the manner described in the investigations on monolayers, and within a few minutes the phase boundary potential acquires the characteristic value of a pancreatin solution (Fig. 11, graph *a*).

In experiments in which equal proportions of pancreatin and egg-albumin are present (0.1 % of each in the solution), the surface rapidly acquires a solid protein film with the characteristic phase boundary potential for egg-albumin (graph *b*); this falls slowly as the concentration of the protein diminishes until what must be regarded as a complex of trypsin and a product of hydrolysis of the protein commences to penetrate the surface layer, gradually increasing in quantity as the protein diminishes until ultimately the surface consists entirely of the trypsin complex possessing phase boundary potentials dependent upon slight variations in the original ratio of enzyme to protein, in general much higher (*e.g.* 178 mv.) than that of a trypsin solution (90 mv. at p_H 8). The surface is, however, perfectly fluid and no change takes place in the phase boundary potential on contracting the surface area by means of a slide. In the last case where the solution contains an excess of protein to pancreatin the surface reaction evidently becomes more complicated (graph *c*). It is observed that the protein film of phase boundary potential 250 mv. forms immediately as in the other cases. The phase boundary potential falls in twenty minutes to 193 mv. and then com-

mences to rise again. The originally rigid gel-like film decreases in rigidity, becomes more elastic and then eventually becomes rigid again. At the end of 220 minutes the original protein-like characteristics of the surface film have all returned.

These films are, however, ultimately digested away after prolonged periods, ca. 10-15 hours.

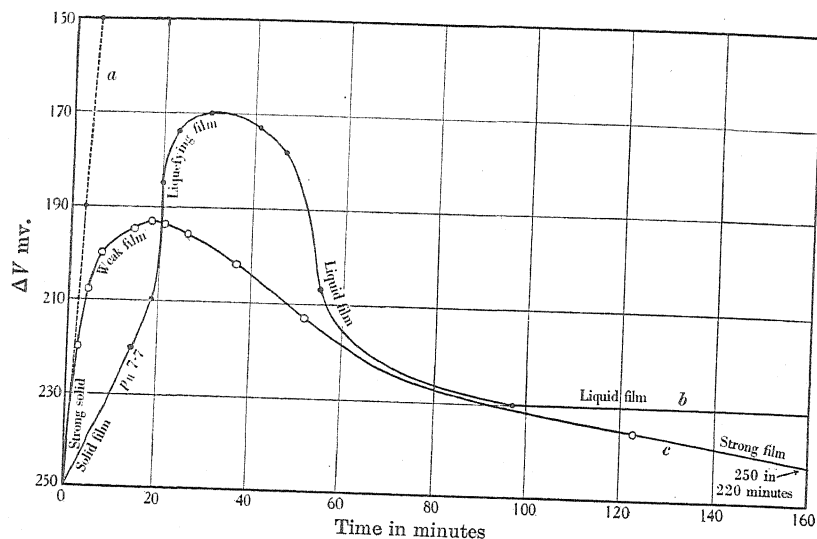


Fig. 11. Surface potential of bulk phosphate at p_H 7.7. *a*. 0.3 g. trypsin + 0.2 g. egg-albumin in 300 cc. at 25°. *b*. 0.3 g. trypsin + 0.3 g. egg-albumin in 300 cc. at 25°. *c*. 0.1 g. trypsin + 0.2 g. egg-albumin in 300 cc. at 25°.

The simplest interpretation to be placed on these observations is that after a preliminary partial digestion of the surface film has taken place the products and the enzyme present near the surface are displaced by the large quantities of undigested protein in the bulk phase and the regenerated protein film then persists until the concentration of the protein in the bulk phase has been greatly reduced.

Further experiments were carried out to test the effects of known by-products in the pancreatin (lipase and amylase) on a protein monolayer.

A pancreatin solution was filtered at p_H 4.6 and the filtrate treated with alumina B. The elution from the alumina containing the lipase and amylase did not influence the surface potential of a caseinogen film, whereas the residue containing the tryptic ferments displaced the caseinogen film in the usual manner.

It may be concluded from these experiments that the change in phase boundary potential and disappearance of a protein film may be taken as an indication that proteolytic hydrolysis or digestion is in reality proceeding.

SUMMARY.

It is shown that when solutions of pancreatin are injected under monolayers of proteins such as egg-albumin or caseinogen proteolytic hydrolysis of the latter proceeds. The reaction can be followed qualitatively visually and quantitatively by observing the concomitant changes in phase boundary potentials.

The reaction kinetics of the proteolytic digestion of protein monolayers follow closely those previously observed for the bulk phase reaction both in respect to dependence on the p_H of the solution and the order of the reaction. It is shown that the proteinase fraction of pancreatin is responsible for the removal of some 10 % of the protein in the case of egg-albumin and of 15 % in the case of caseinogen, whilst the carboxypolypeptidase fraction removes a further 25 and 16 % respectively.

The proteinase and carboxypolypeptidase fractions of pancreatin when purified by the precipitation or by the adsorption process form films which are protein-like in character. These are themselves capable of being hydrolysed by pancreatin without loss of surface activity.

The characteristics of films of trypsin are found to be indistinguishable from those of albumin over the region of p_H in which they exhibit no tryptic activity, whilst over the active region they form solutions of low surface phase boundary potentials.

It is shown that when pancreatin solutions are digested by pepsin at p_H 2 and the solution restored to p_H 8, whilst no tryptic activity is observed for bulk phase reaction, monolayers of egg-albumin and caseinogen are readily digested. Solutions of trypsin when boiled at p_H 8 likewise exhibit a marked surface proteolytic activity.

The properties of the surfaces of solutions containing both enzyme and protein are examined during the course of the reaction. It is concluded that in the tryptic enzymes the "free enzymes" can be separated from their protein-like carriers but when free can only react with proteins when presented to them in a suitable form such as a monolayer at an air-water interface. To render the "free enzyme" active for protein solutions it must be adsorbed on a protein in a particular state or configuration. Experimental evidence is given that the disappearance of a protein film on an enzyme containing substrate is due to digestion only and is not due to the adsorption of some constituent of the enzyme of lower surface tension.

We wish to express our thanks both to Prof. Northrop of Princeton and to D. R. P. Murray for their kindness in furnishing us with specimens of the purified enzymes and to the latter for assistance in carrying out bulk control experiments; to Dr J. Zozoya for much helpful criticism and to the Medical Research Council for a grant to one of us (J. S.).

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CCXVIII. FERMENTABLE SUGAR IN FASTING URINE.

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(Received August 11th, 1933.)

THERE seems no doubt that the post-prandial urine of normal men and women often contains a reducing substance or substances removable by yeast. This "fermentable sugar" has been found after breakfast by Van Slyke and Hawkins [1929], at irregular intervals during the day both by Greenwald *et al.* [1927] and by Harding and Selby [1931], and in 24 hour urines by West and Peterson [1932]. Its occurrence varies with the nature of the meal (Harding and Selby) or the general diet [West, Lange and Peterson, 1932] and with the individual (Harding and Selby). Whilst all the methods for its determination have in common the use of yeast, different reagents have been used for the determination of the reducing power, thus minimising the possibility that the findings are due to some extraneous accident of technique. The fraction removable by yeast almost certainly contains glucose. The fraction is found in the carbohydrates of the urine. Harding and Selby provided physiological evidence for the entry of glucose from the blood, and West and Steiner [1932] state that the rate of fermentation is almost exactly paralleled by the same amount of glucose under the same conditions. On the other hand Greenwald *et al.* believed it to be non-glucose in character, and Van Slyke and Hawkins have clearly shown the simultaneous occurrence of small amounts of irregularly excreted non-glucose substance removable by yeast. Harding and Selby also showed the possible occurrence of fructose.

The previous paper of Harding and Selby reported the practical absence of a substance removable by yeast from fasting urines and at many other times during the 24 hours. We did not deny the total absence of glucose, but recognising the limitations of the method of analysis, regarded such amounts of that sugar as might be present in these urines as minimum. Certainly there was no difficulty in differentiating between these possible minimum amounts and the comparatively large excretion of post-prandial substances removable by yeast. This irregularity of excretion, affecting both the time of day and the individual, enabled the authors to harmonise their own results with the previous contradictory findings reported in the literature.

In a recent series of papers West and his colleagues [West and Peterson, 1932; West, Lange and Peterson, 1932; West and Steiner, 1932] claim that fermentable sugar occurs in all urines. This they identify as glucose.

The crucial point of difference between ourselves and West *et al.* lies in the possible amount of fermentable sugar in fasting urine. West and Peterson ascribe our lack of ability to find fermentable sugar in fasting urines to the operation of a number of causes which, acting together, reduce the effective amount of this substance to such an extent that the titration difference between the total sugar and non-fermentable sugar becomes indistinguishable from the

titration error. They calculate that the presence of 6 mg./100 cc. fermentable sugar in urine at a dilution 1:10 would only mean a titration difference of 0.08 cc. 0.005 *N* thiosulphate under our conditions, and a difference of 0.04 cc. at dilution 1:20. West, Lange and Peterson go so far as to remark "that the method used by these workers is incapable of estimating with sufficient accuracy the fermentable sugar of starvation urines."

In our previous paper we drew the conclusion that "the possible amounts of fermentable sugar in normal fasting urine must be under 5 mg./100 cc. and are more likely to be less than 2-3 mg./100 cc." The results on which this conclusion is based are to be seen in Tables I and II of the previous paper. In Table I, where the dilution is constant at 1:10, only 1 urine out of 8 shows a titration difference of 0.04 cc. Evidently in this series of urines, the amount of fermentable sugar is 3 mg./100 cc. or less, and such a conclusion neglects the fact that some of the urines gained slightly in reducing power, rather than lost. In Table II the dilutions are varied, but only 4 out of 18 urines show fermentable sugar equal to 0.03 cc. 12 out of the 18 show titration differences of +0.01 or 0.00 cc. The average result lies within our experimental error, and we consider our conclusion justified. Our conclusions were supported also by an unpublished series of observations on 8 normal fasting urines of which we now give the details of 4. The methods were those of our previous paper.

Extreme care was taken in this series. All apparatus was recalibrated and especially cleaned before use. The yeast was most thoroughly washed and after use on the diluted urine was removed by a double centrifuging. Two samples from each urine specimen were analysed separately, with duplicate titrations on each. The extreme range of all 4 titrations did not exceed 0.02 cc. 0.005 *N* thiosulphate. The average value thus only varies from the extreme by ± 0.01 . Table I shows the results of 2 urines diluted 1:10 and 2 diluted 1:20. The amount of sugar removable by yeast is represented by differences of ± 0.01 cc.

Table I.

Subject	Specimen	Final dilution	Total sugar	Non-fermentable sugar	Fermentable sugar
			cc. 0.005 <i>N</i> thiosulphate per 2.0 cc. filtrate		
I	1	1 : 10	1.02	1.03	
			1.02	1.02	
	2		1.01	1.03	
			<u>1.02</u>	<u>1.01</u>	
	Average	1.02	1.02	0.00	
II	1	1 : 10	0.83	0.84	
			0.83	0.83	
	2		0.82	0.84	
			<u>0.83</u>	<u>0.84</u>	
	Average	0.83	0.84	- 0.01	
III	1	1 : 20	0.57	0.55	
			0.57	0.54	
	2		0.55	0.55	
			<u>0.56</u>	<u>0.55</u>	
	Average	0.56	0.55	+ 0.01	
IV	1	1 : 20	0.66	0.64	
			0.64	0.65	
	2		0.65	0.65	
			<u>0.64</u>	<u>0.64</u>	
	Average	0.65	0.65	0.00	

It would seem a justifiable conclusion that fermentable sugar, or sugar removable by yeast, was present in these urines only in minimum amounts. Had we been dealing with aqueous solutions of glucose we should have detected with certainty 1-2 mg./100 cc. With urines the sensitivity of our reagents appeared somewhat less, and we set the limits of detection at 2-3 mg./100 cc. Our reagents and methods were of sufficient delicacy to detect glucose in the concentrations stated by West *et al.*

So far neither West *et al.* nor ourselves have confirmed the presence in urine of the non-glucose reducing substance removable by yeast of Van Slyke and Hawkins. These latter obtained evidence for its existence in urines diluted only 1:1, when acted on by an equal volume of washed centrifuged yeast cells. There was possibly some indication of its existence in the urine No. 1 in Table I of our previous paper, where 3 successive treatments with yeast removed equivalents of 0.02, 0.03 and 0.03 cc. 0.005 *N* thiosulphate. The amount was very small, so small that we felt no correction was required in the majority of urines. Nevertheless we minimised the possible interference of this substance by using a highly diluted urine. At the same time in our study of post-prandial urines we refused to consider as true fermentable sugar any difference less than 0.05 cc. 0.005 *N* thiosulphate. The use of urines of only moderate dilution, and the interpretation of very small diminutions in reducing power after the action of yeast as true fermentable sugar, were open to the criticism that we were dealing with the Van Slyke-Hawkins or a similar substance, which when present in very small amounts became completely removable by a single yeast action.

Although as a result of West's criticism we have felt ourselves under the necessity of defending and amplifying our previous work, nevertheless it is a matter of surprise that there is any serious matter for disagreement. Both laboratories use the same general method of analysis, the yeast preparations and their use are the same, and the copper reagents differ only in a few details. Both methods quantitatively recover added glucose, either as a direct addition to the total sugar value, or as extra fermentable sugar. If the fermentable sugar found by West *et al.* in fasting human urine consists entirely of glucose, the two sets of observations should be in substantial, if not complete, agreement. True, West uses $\text{HgSO}_4\text{-BaCO}_3$ in his preliminary treatment of the urine and we have used $\text{H}_2\text{SO}_4\text{-Lloyd's reagent}$. This however should affect only the level of the total sugar and non-fermentable sugar determinations and not their difference, if glucose is the sole urinary fermentable sugar. The present paper contains the results of some observations directed towards discovering if possible the cause of the discrepancies.

GENERAL METHODS.

The general method of analysis is the same as in our previous paper. After dilution the urine is treated with a suitable reagent to remove interfering substances. The total "sugar" is determined on 2 cc. filtrate. Another portion of the diluted urine (10-15 cc.) is treated with 0.25 g. wet-weight washed centrifuged yeast for 8 minutes at 37° with constant stirring. The yeast is removed by centrifuge. The non-fermentable "sugar" is determined on 2 cc. of this centrifugate after clearing. All thiosulphate titrations were performed in triplicate. Two copper reagents have been used. The first is that described by Harding and Selby [1931] and the second is that described by Harding and Downs [1933]. The chief difference between them is the carbonate-bicarbonate ratio. We shall refer to these reagents as 1 and 2.

All urine-filtrates were adjusted to p_{H} 6.5 previous to "sugar" determinations.

Influence of ammonia on "sugar" determinations.

Harding and Downs [1933] showed that very small concentrations of ammonium salts decreased the sensitivity of their reagent to sugars in aqueous solution. The removal of ammonia could readily be effected by the addition of KH_2PO_4 followed by solid MgO . This gave a filtrate containing the original amount of sugar. The presence of ammonium salts in urines may thus cause a certain amount of error in sugar determinations, unless the preliminary "clearing" at the same time removes the ammonia. In normal urines the amount is not large, and the error, in view of the uncertainty of interpretation to be attached to total sugar determinations, is probably not serious. The use of $\text{HgSO}_4\text{-BaCO}_3$ as recommended by West gives a filtrate free from NH_3 . Lloyd's reagent leaves some NH_3 , but this clearing agent can be preceded or followed by the $\text{KH}_2\text{PO}_4\text{-MgO}$ treatment.

However great or small may be this possible source of error on the determination of total sugar, within the limits of its normal variation, the presence of NH_3 has no effect on the yeast action. There thus appears to be no reason to suspect an inaccuracy of our previous determinations of fermentable sugar from this cause.

A fasting 2 hour urine sample was diluted to 150 cc. To 120 cc. of the diluted urine were added 1.2 g. KH_2PO_4 and 2.4 g. solid light calcined MgO . After shaking 15 minutes the mixture was filtered and the filtrate found NH_3 -free by Nessler's reagent. The filtrate was brought to p_{H} 6.5 by adding 1 or 2 drops of 20 % H_2SO_4 . 100 cc. of 0.04N H_2SO_4 were added to 100 cc. urine-filtrate and 6 g. Lloyd's reagent. After shaking a few minutes, the treated urine was filtered. A determination of total sugar and non-fermentable sugar after yeast action was made. To 50 cc. of the cleared diluted urine were added 50 mg. $(\text{NH}_4)_2\text{SO}_4$. This represents approximately 150 mg. NH_3 per 100 cc. original urine. Total sugar and non-fermentable sugar after yeast action were again determined. The added NH_3 was removed from the remainder of the liquid by KH_2PO_4 and MgO . Total sugar and non-fermentable sugar after yeast action were again determined.

The sugar estimations were made by both copper reagents. The results, shown in Table II, demonstrate the very slight effect of the added ammonium salt when the estimations were made by reagent 1 and the marked effect on reagent 2. The effect, however, is the same on both total sugar and non-fermentable sugar determinations. The yeast action is evidently unaffected.

Table II. *Showing the action of added NH_3 on total sugar and non-fermentable sugar determinations and the accuracy of fermentable sugar determinations in presence of ammonium salts.*

Procedure on urine	"Sugar" as cc. 0.005N thiosulphate per 2 cc. filtrate					
	Reagent 1			Reagent 2		
	T.S.	N.F.S.	F.S.	T.S.	N.F.S.	F.S.
$\text{KH}_2\text{PO}_4\text{-MgO}$ and $\text{H}_2\text{SO}_4\text{-Lloyd's reagent}$	2.82	2.44	0.38	2.07	1.77	0.30
As above + added $(\text{NH}_4)_2\text{SO}_4$	2.80	2.42	0.38	1.75	1.45	0.30
Added $(\text{NH}_4)_2\text{SO}_4$ removed by $\text{KH}_2\text{PO}_4\text{-MgO}$	2.82	2.44	0.38	2.07	1.76	0.31

T.S. = Total sugar.

N.F.S. = Non-fermentable sugar.

F.S. = Fermentable sugar.

The use of $\text{KH}_2\text{PO}_4\text{-MgO}$ supplies another method for "clearing" urines previous to sugar determinations. This method removes very little reducing material, giving high "sugar" values when used alone. Such urines are Ca-free and contain only a small amount of phosphate.

Effect of variations in methods of clearing of urine and different copper reagents.

If the directions of West and Peterson for the use of $\text{HgSO}_4\text{-BaCO}_3$ are followed and the interfering substances of urine removed by this reagent, there is no difficulty in finding fermentable or yeast-removable sugar in every fasting urine we have examined, at dilutions varying from 1:5 to 1:15 and using 2 cc. filtrate for the titrations. We have found it convenient to express our results in mg. glucose per 2 hours, but assuming a regular excretion throughout the day the amount would vary from 36 to 60 mg. The concentration varies from 4 to 15 mg./100 cc. The yeast was used on diluted urine and previous to the $\text{HgSO}_4\text{-BaCO}_3$ treatment.

If the diluted urine is treated with $\text{KH}_2\text{PO}_4\text{-MgO}$ followed by $\text{H}_2\text{SO}_4\text{-Lloyd's}$ reagent, following directions similar to those given in the previous section, and the yeast action is allowed to take place just previous to the treatment with $\text{H}_2\text{SO}_4\text{-Lloyd's}$ reagent, a series of figures is obtained for fermentable sugar similar to those obtained by the use of $\text{HgSO}_4\text{-BaCO}_3$. Both results are independent of the copper reagent.

If the treatment or "clearing" of the urine is solely by $\text{H}_2\text{SO}_4\text{-Lloyd's}$ reagent, two sets of results are obtained for fermentable sugar. The determination of total and non-fermentable sugar by reagent 1 (the reagent used in our previous paper) gives figures for fermentable sugar approximately one-half of those given by reagent 2, or by either reagent after the use of $\text{HgSO}_4\text{-BaCO}_3$ etc. The fermentable sugar is now 1 to 3 mg. per 2 hours, reckoned as glucose, and its concentration varies from 2 to 6 mg./100 cc.

All these results are shown in Table III. The values for fermentable sugar are obtained by subtraction of the non-fermentable sugar from the total sugar values in mg./2 hours and not by calculation from the difference as given in cc. 0.005N thiosulphate. This causes occasional slight discrepancies between

Table III. *Showing the effect of different clearing reagents on the estimation of total (T.S.), non-fermentable (N.F.S.) and fermentable (F.S.) sugar.*

Figures represent mg./2 hours as glucose. Bracketed figures are cc. 0.005N thiosulphate for 2 cc. urine filtrate.

Subject	Vol./ 2 hours cc.	Approx. final dilution	$\text{H}_2\text{SO}_4\text{-Lloyd's}$ reagent			$\text{KH}_2\text{PO}_4\text{-MgO}$ and $\text{H}_2\text{SO}_4\text{-Lloyd's}$ reagent			$\text{HgSO}_4\text{-BaCO}_3$			Copper reagent
			T.S.	N.F.S.	F.S.	T.S.	N.F.S.	F.S.	T.S.	N.F.S.	F.S.	
A.R.A.	34	1:15	36	34	2 (0.06)	40	35	5 (0.13)	24	20	4 (0.11)	1
			28	24	4 (0.15)	28	23	5 (0.15)	21	16	5 (0.15)	2
T.F.N.	94	1:5	49	47	2 (0.05)	51	46	5 (0.15)	30	25	5 (0.14)	1
			32	28	4 (0.13)	35	30	5 (0.16)	25	20	5 (0.17)	2
D.L.S.	55	1:10	50	48	2 (0.04)	50	45	5 (0.12)	27	24	3 (0.09)	1
			31	29	2 (0.09)	33	30	3 (0.11)	23	19	4 (0.12)	2
G.A.G.	56	1:10	54	53	1 (0.03)	52	48	4 (0.13)	30	25	5 (0.14)	1
			37	33	4 (0.12)	37	33	4 (0.12)	26	21	5 (0.12)	2
S.J.	112	1:5	50	47	3 (0.07)	48	43	5 (0.14)	28	24	4 (0.10)	1
			32	28	4 (0.13)	33	28	5 (0.15)	28	24	4 (0.17)	2
C.E.D.	57	1:10	43	41	2 (0.06)	48	42	6 (0.18)	25	21	4 (0.10)	1
			27	22	5 (0.16)	31	26	5 (0.17)	20	16	4 (0.15)	2
C.E.D.	50	1:10	42	40	2 (0.07)	46	40	6 (0.17)	23	19	4 (0.10)	1
			30	26	4 (0.14)	33	28	5 (0.18)	21	17	4 (0.15)	2

the values as shown in the two columns, as we have approximated the values for total and non-fermentable sugar to the nearest mg.

Two questions at once arise. Why does copper reagent 1 compared with reagent 2, and used after H_2SO_4 -Lloyd's reagent, estimate only one-half the fermentable sugar, and why do the results not agree with those of Tables I and II of our previous paper and Table I of the present paper? To the first question it would seem a reasonable answer that reagent 2 determines some non-glucose substance removable by yeast which is estimated either partially or not at all by reagent 1. If so, this substance might have been altered by the previous treatment of the urine by KH_2PO_4 -MgO, for the fermentable sugar is now the same by both reagents. Treatment of urine with HgSO_4 - BaCO_3 apparently also alters its value to the same degree, unless the agreement is entirely fortuitous. It must be remembered that the yeast action in our experiments has taken place before the treatment with H_2SO_4 -Lloyd's reagent or HgSO_4 - BaCO_3 , so that any change should be shown as an apparent increase in total sugar. Of the two copper reagents No. 2 was designed to have a very much smaller oxidising power on some nitrogenous reducing substances, and HgSO_4 - BaCO_3 was designed by West to remove a number of such possible substances. Changes of this character overshadow to such an extent any possible alterations in the reducing power of a small amount of non-glucose substance removable by yeast that no direct indication of the existence of this latter can be obtained from our data. The facts however are inconsistent with glucose being the sole fermentable or yeast-removable substance in urine. The second question is discussed later.

Recovery of added glucose from urine.

In view of the inconsistencies just reported we feel it necessary to give the actual recovery figures of small amounts of glucose from urine in which the clearing agent and the method of using the yeast are varied. Eagle [1927] had advised the use of yeast after H_2SO_4 -Lloyd's reagent. Usually we have used yeast before the clearing agent. We determined added glucose when approximately 2.5 mg. glucose were added to a 2 hour fasting urine specimen and the "clearing" was varied as follows:

- I. urine diluted and "cleared" by H_2SO_4 -Lloyd's;
- II. urine diluted and "cleared" by KH_2PO_4 -MgO;
- III. urine diluted and "cleared" by KH_2PO_4 -MgO followed by H_2SO_4 -Lloyd's reagent;
- IV. urine diluted and "cleared" by HgSO_4 - BaCO_3 .

Table IV. *Showing recovery of small amounts of glucose added to normal fasting urine. The glucose is recovered as extra fermentable sugar.*

Figures represent mg./2 hours.

Method	Yeast used previous to "clearing"				Yeast used after "clearing"			
	Fermentable sugar present in urine Reagent		Glucose added 2.6 mg. Extra fermentable sugar Reagent		Fermentable sugar present in urine Reagent		Glucose added 2.7 mg. Extra fermentable sugar Reagent	
	1	2	1	2	1	2	1	2
I	4.4	3.0	2.8	3.1	3.2	4.2	2.9	2.6
II	3.6	2.6	3.4	2.8	5.5	3.8	2.8	2.8
III	3.8	3.8	2.9	2.8	3.4	4.9	3.4	2.2
IV	3.0	3.7	2.5	2.8	3.4	4.7	3.1	2.4

The preliminary dilution was approximately 1 : 3. The yeast was used on the preliminary diluted urine, before the clearing agent in one series of experiments and after the clearing agent in a second series. The final dilution was 1 : 6. The reducing power was determined by both copper reagents. All methods of clearing and variations in the use of yeast gave satisfactory recovery figures (Table IV).

Effect of dilution preliminary to clearing.

We have stated that to minimise in our previous experiments the effect of any possible non-glucose substances removable by yeast on our determinations of fermentable sugar in normal urine, we used a high dilution. Apart from the limits of our ability to estimate glucose, to what extent did the high preliminary dilution contribute to our previous negative findings?

A. A 2 hour specimen of normal fasting urine was diluted to 150 cc. (dilution approximately 1 : 3). On a portion of this were determined total and non-fermentable sugar, using H_2SO_4 -Lloyd's reagent and allowing the yeast to act previous to clearing. The final dilution, at which the sugar determinations were made, was approximately 1 : 6. Except for a more concentrated urine, the technique follows exactly that of our previous paper.

A/3. Portions of the final 1 : 6 dilution urines from A were diluted with water to a dilution 1 : 18.

B. 50 cc. of the preliminary diluted (1 : 3) urine from A were diluted to 150 cc., making a new preliminary dilution of 1 : 9. On this were determined total sugar after H_2SO_4 -Lloyd's reagent and non-fermentable sugar after yeast and H_2SO_4 -Lloyd's reagent. The final dilution was 1 : 18. Copper reagents 1 and 2 were used in the three sets of determinations.

A comparison of the results from A, A/3 and B should show the same amount of fermentable sugar in mg./2 hours if glucose is the sole fermentable sugar in urine. Expressed in cc. 0.005*N* thiosulphate per 2 cc. final dilution of urine, the fermentable sugar of both A/3 and B should be 1/3 the value of A, if we are dealing only with glucose. If, however, substances other than glucose constitute part of this fraction, the values for fermentable sugar in B might vary markedly from those of A/3.

The results are shown in Table V. The amount of fermentable sugar found in B is very much less than in either A or A/3. With reagent 1 the amount is less than one-half, in all but one sample. The use of reagent 2 shows 4 samples of B out of 6 to contain less fermentable sugar than A. The comparison of A and A/3 shows the latter in cc. 0.005*N* thiosulphate to be almost exactly 1/3 the former.

Table V. *Showing the effect of the amount of dilution preliminary to the action of yeast and H_2SO_4 -Lloyd's reagent on fermentable sugar in normal fasting urine.*

Subject	Copper reagent 1			Copper reagent 2		
	A	A/3	B	A	A/3	B
R.W.I.U.	0.31	0.10	0.09	0.32	0.12	0.07
C.E.D.	0.28	0.10	0.04	0.30	0.10	0.09
C.E.D.	0.33	0.12	0.02	0.27	0.09	0.06
R.A.	0.40	0.13	0.06	0.41	0.12	0.08
D.L.S.	0.25	0.08	0.07	0.23	0.08	0.05
G.A.G.	0.42	0.12	0.04	0.27	0.08	0.10
Average	0.33	0.11	0.05	0.30	0.10	0.07
mg./2 hours as glucose	7.0	7.0	3.2	5.2	5.2	3.6

Results in cc. 0.005*N* thiosulphate per 2 cc. urine-filtrate.

The error of simple dilution is 0.01 cc. Again the facts are inconsistent with the unity of the fermentable sugar fraction of normal fasting urine.

Why do the results with reagent 1 in the present work fail to duplicate our previous results, and why does reagent 1 give lower, similar and higher results with reagent 2 in Tables III, IV and V? Whilst we have not entirely excluded the possibility that small unconscious differences in detail in the use of the copper solutions or the preparation of the filtrates may be responsible for the discrepancies, it seems more likely that inequalities in the preparation of reagent 1 are the cause. The figures for fermentable sugar in fasting urine as obtained by reagent 2 are relatively stable from preparation to preparation, and each preparation can be easily checked by reference to the p_H or titration values of the B solution (Harding and Downs). Reagent 1 has also been standardised as far as possible, and each preparation is always checked in value against pure glucose and found to give the same standard figure. This, however, is known to be no guarantee that each preparation will possess the same value to all other reducing substances. Harding and Van Nostrand [1930] noted variations in oxidising power to non-sugar reducing substances of whole blood. Harding and Selby drew attention to the same fact, and this instability of the preparation was one of the reasons that caused us to seek a different copper oxidising reagent with good general properties. The instability is brought about in the preparation of the reagent by the necessity of neutralising Na_2CO_3 by free tartaric acid, with a resultant uncertainty of the carbonate-bicarbonate ratio, though this can be controlled with sufficient accuracy to obtain constant results with glucose (see criticisms by Somogyi [1926]). The results for fermentable sugar in normal fasting urine and after glucose, both morning and afternoon, as reported by Harding and Selby in their previous paper, were all obtained with one large preparation of the copper reagent. Two or three preparations have been used in the work of the present paper. If our explanation is correct, a somewhat accidental circumstance combined with our high preliminary dilution was responsible for our previous negative findings for fermentable sugar in normal fasting urine. On the other hand, all the evidence of the present paper makes uncertain the amount of glucose present in such urines and favours the minimum rather than the maximum figures.

Effect of oral administration of glucose on urine "fermentable" sugar.

We have re-examined this question, using conditions favourable to showing a high sugar removal by yeast.

50 g. glucose in 400 cc. water were taken orally at 9 a.m. under the same conditions as in our previous paper. Some days later 50 g. glucose were taken by the same subjects at 4 p.m. (under conditions of afternoon glycosuria), the noon meal being of general mixed character. Blood-sugars were estimated at 9 a.m., 2 p.m. and 4 p.m., and every 15 minutes after the glucose administration. We have reported only the zero, peak and 2 hour values. All blood-sugars were determined on cutaneous blood [Herbert and Bourne, 1930]. Urine was cleared by KH_2PO_4 -MgO followed by H_2SO_4 -Lloyd's reagent, and also by $HgSO_4$ - $BaCO_3$. Yeast was used after clearing and the reducing power determined by reagent 2. The final dilution was approximately 1 : 10.

Table VI shows that there is a small rise in urine "fermentable" sugar after oral glucose administration, whether given fasting at 9 a.m. or at 4 p.m. even when the peak of the blood-sugar does not rise above the normally accepted renal threshold. When the peak rises above the usual threshold value, as in cases C.E.D. and A.R.A., the usual large amounts of fermentable sugar enter the urine. Unless this latter occurs, the increases, however, are so small that

Table VI. *Showing effect of oral glucose on urine "fermentable" sugar.*

Subject	Morning or afternoon	Blood-sugar, mg./100 cc.			Urine "fermentable" sugar mg./2 hours	
		0 hour	Peak	2 hours	Pre-glucose	Post-glucose
D.L.S.	M	77	124	97	5.0 3.5	6.4 6.8
	A	94	144	81	11.0 10.7	11.4 11.1
T.F.N.	M	87	130	85	3.8 —	4.5 —
	A	107	173	94	9.2 10.2	14.6 16.0
S.J.	M	80	132	66	2.7 3.5	7.0 6.1
	A	93	149	109	4.6 5.6	7.1 7.3
G.A.G.	M	90	134	82	5.8 5.5	7.0 6.7
	A	86	173	87	9.1 10.4	13.2 13.9
A.R.A.	M	107	150	60	4.7 5.0	6.1 6.1
	A	92	232	123	2.4 2.6	317 317
C.E.D.	M	97	154	91	4.1 4.4	5.2 5.0
	A	105	204	105	5.7 8.0	338 343

The first of each pair of urine "fermentable" sugar values is obtained after $\text{KH}_2\text{PO}_4\text{-MgO}$ followed by $\text{H}_2\text{SO}_4\text{-Lloyd's}$ reagent. The second of each pair is after $\text{HgSO}_4\text{-BaCO}_3$.

our previous conditions would have failed to detect them, unless we had adopted the extremely rigorous technique of Table I.

A correspondence between the blood-sugar level and the amount of urine "fermentable" sugar as determined after $\text{HgSO}_4\text{-BaCO}_3$ or $\text{KH}_2\text{PO}_4\text{-MgO}$ and $\text{H}_2\text{SO}_4\text{-Lloyd's}$ reagent is also shown in Table VII. The morning blood-sugar

Table VII. *Showing relation of fasting blood-sugar to level of urine "fermentable" sugar.*

Subject	Blood-sugar 9 a.m. mg./100 cc.	Urine-sugar 7-9 a.m. mg./2 hr.	Blood-sugar 2-4 p.m. mg./100 cc.	Urine-sugar 2-4 p.m. mg./2 hr.
D.L.S.	77	5.0* 3.5†	115-94	11.0* 10.7†
S.J.	80	2.7* 3.5†	88-93	4.6* 5.6†
T.F.N.	87	3.8* —	122-107	9.2* 10.2†
G.A.G.	90	5.8* 5.5†	117-86	9.1* 10.4†
C.E.D.	97	4.1* 4.4†	124-105	5.7* 8.0†
A.R.A.	107	4.7* 5.0†	83-92	2.4* 2.6†

* Urine "cleared" by $\text{KH}_2\text{PO}_4\text{-MgO}$ followed by $\text{H}_2\text{SO}_4\text{-Lloyd's}$ reagent.

† Urine "cleared" by $\text{HgSO}_4\text{-BaCO}_3$.

at 9 a.m. is assumed as the level from 7-9 a.m. [Trimble and Maddock, 1929]. The blood-sugar level at 2 p.m. and 4 p.m. is usually higher than the fasting morning level. In all these cases the urine "fermentable" sugar is higher from 2-4 p.m. than from 7-9 a.m. The one exception where the 2-4 p.m. blood-sugar level is lower than in the morning shows a lower urine "fermentable" sugar.

The conclusion must be reached that fermentable sugar as obtained under such conditions is intimately connected with the level of the blood-sugar, or with the level of carbohydrate metabolism of which the fasting and post-prandial blood-sugar is also a reflex. In view of the facts set forth in the preceding sections showing the lack of unity of proof that glucose is the sole constituent of the fraction called urine "fermentable" sugar, we hesitate to conclude that the increases are glucose. The hesitation is strengthened if we tabulate the increases in urine "fermentable" sugar after glucose feeding in the order of the peaks of the blood-glucose. We should expect the two sets of figures to show some degree of correspondence if there is a simple escape of blood-glucose through the kidney. Table VIII shows such an arrangement. In making up the table

Table VIII. *Showing the peaks of blood-sugar and increase in urine "fermentable" sugar after glucose feeding.*

Blood-sugar peak mg./100 cc.	Urine "fermentable" sugar		
	Pre-glucose mg./2 hours	Post-glucose mg./2 hours	Increase
124	4	6	2
130	4	4	0
132	3	6	3
134	6	7	1
144	11	11	0
149	5	7	2
150	5	6	1
154	4	5	1
173	9	13	4
173	9	15	6
204	7	340	333
232	2	317	315

we have taken the mean of the $\text{KH}_2\text{PO}_4\text{-MgO}$ and the $\text{HgSO}_4\text{-BaCO}_3$ values as representing the urine "fermentable" sugar to the nearest mg. It will be seen that no close correspondence between increases in blood-sugar and urine "fermentable" sugar exists. The increases after glucose feeding, though distinct, are small and remain so until, in this small series, the peak of the blood-sugar reaches 170 mg. The increase at this point is much more marked than after the lower blood-sugar peaks. At a peak of 204 mg. or 232 mg. the urine becomes flooded with fermentable sugar. This is the familiar description of the renal threshold for glucose, with a slight variation. Our figures supply no evidence for a gradual progressive increase in urine "fermentable" sugar with increasing peaks in blood-glucose.

West, Lange and Peterson evidently intend it to be understood that urine "fermentable" sugar as determined by their method is dependent on the level of the blood-sugar. They draw attention to a sharp increase in fermentable sugar in a dog's urine occurring after meat feeding and remark, "it is probable also that the glucose of the blood reached a maximum about this time." Yet they ignore their findings that extremely irregular excretions of fermentable sugar apparently occur on two successive days of starvation in the same individual. Subject E.S.W. excretes 326 mg. fermentable sugar on normal diet, then 308 mg.

and 68 mg. on two starvation days. Subject H.L.W. puts out 38 mg. and 160 mg. fermentable sugar on two successive starvation days, the latter being slightly greater than after a day's normal diet. Such findings are inconsistent with a close correlation of blood-sugar and urine "fermentable" sugar. The blood-sugar during fasting exhibits no large fluctuations, such as are produced by the intake of food. Sugar remains at the normal fasting level with a slow fall as the fasting progresses from day to day.

SUMMARY.

1. Ammonium salts, in physiological amounts, lower the total "sugar" and non-fermentable "sugar" found in urine, when the determinations are made by some of the sensitive Shaffer-Hartmann reagents. The fermentable sugar value may be unaltered.
2. The use of KH_2PO_4 followed by MgO on normal urines constitutes a method of clearing urine. The filtrates are suitable for "sugar" determinations.
3. KH_2PO_4 - MgO can be used in conjunction with H_2SO_4 -Lloyd's reagent for clearing urines previous to "sugar" determinations.
4. The values for fermentable sugar in normal fasting urine can vary with the clearing agent and with the copper reagent.
5. All the variations of method permit of the recovery of added glucose to urine, in the form of extra fermentable sugar.
6. The amount of fermentable sugar found in normal fasting urine can be varied by the state of dilution previous to the application of H_2SO_4 -Lloyd's reagent.
7. Provided the method of clearing and the oxidising reagent correctly estimate small amounts of added glucose, the lower values for fermentable sugar should be accepted as more nearly correct.
8. Using the methods which give the higher results for fermentable sugar in urine, the oral ingestion of glucose results in slight but distinct increases in this fraction. These increases cannot be correlated with the increase in the blood-sugar. When the usual renal threshold for sugar is passed, large amounts of sugar appear in the urine.
9. We conclude that some substance removable by yeast other than glucose exists in normal human fasting urine.

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CCXIX. THE EFFECTS OF AMINES ON OXIDATIONS OF THE BRAIN.

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(Received August 29th, 1933.)

AN investigation into the effects of narcotics on the oxidative processes of the brain has shown [Quastel and Wheatley, 1932, 1, 2] that these substances exert specific inhibitions at low concentrations. The oxidations of glucose, sodium lactate and sodium pyruvate are markedly affected whilst those of sodium succinate and *p*-phenylenediamine are unimpaired. This generalisation holds for all narcotics investigated, the inhibitory power varying, among narcotics of the same chemical type, with the hypnotic activity. A view of the mechanism of narcosis, which fits the observed facts, is that the narcotic is adsorbed from the blood-stream at a specific area or centre of the nervous system. Here it diminishes the ability of the cells constituting the nervous centre to activate lactic or pyruvic acid and in this way inhibits the oxidation by these cells of glucose, lactic or pyruvic acid. The activation and access of oxygen remain unimpaired, but, in consequence of the inhibition of oxidative powers, a lowered amount of energy is available to the narcotised nervous centre for the accomplishment of its functional activity. Narcosis—or a depression of the normal activity of the centre in question—may then ensue. In spite therefore of the fact that oxygen is freely available, a state equivalent to partial anoxaemia exists at those areas of the nervous centre where the narcotic is adsorbed. The results of experiments carried out under anaerobic conditions (oxygen being replaced by methylene blue) show that the adsorption of the narcotic is reversible, a competition taking place between lactic acid and the narcotic for possession of the active surfaces in the nervous centre [Davies and Quastel, 1932].

A development of this work now shows that there exist substances, normally produced in the body, whose effects on the oxidative processes of the brain closely resemble those of the narcotics. This result is of particular significance in the study of mental disorder. Recent investigations [McFarland, 1932] have indicated that the psychological reactions following anoxaemia and light narcosis closely resemble those found in certain psychotic disorders. It is not unreasonable to consider that if substances normally produced in the body have effects similar to those of the narcotics and can induce an anoxaemia in the nervous system, they may be held, when present in the blood-stream in more than ordinary quantities, partially responsible for psychotic manifestations.

The substances in question have been found to be chiefly degradation products derived from tyrosine and tryptophan.

Experimental method.

The technique employed in this investigation has been described fully in previous papers by the writers [1932, 1, 2] in which all details are available. The method consists, briefly, in allowing mixed (whole) brain tissue, usually of the guinea-pig, to take up oxygen at 37° in a Barcroft respirometer until the rate of uptake has fallen to between 50 and 75 % of the initial value. After this period (about 2½ hours) glucose, sodium lactate or other metabolite under investigation is added to the tissue and the new rate of oxygen uptake determined. This rate is compared with that obtained by brain tissue exposed from the commencement of the experiment to the substances whose narcotic effects are the subject of this study.

The experiments are carried out on 0.5 g. tissue (made as homogeneous as possible by chopping with a scalpel) in a saline-phosphate (*M*/15) medium at an initial p_H of 7.4. The amines *etc.* are dissolved in saline and brought to neutrality before adding to the brain tissue. Measurements of O_2 uptake are made at 15 minute intervals, all the Barcroft vessels containing air. Experiments have been carried out with brain (cortex) slices in an atmosphere of oxygen, but, since it appears a characteristic feature of brain that mincing or chopping in the manner described does not appreciably affect its oxidative mechanisms (so far as the substances mentioned in this paper are concerned) and since the magnitude of the Q_{O_2} of brain slices (in oxygen) is much the same as that of minced brain (in air), the writers have confined themselves almost exclusively to work with the minced tissue. The results which have been obtained with brain slices are in agreement with those with the minced tissue.

Results.

The amounts of oxygen consumed by 0.5 g. guinea-pig (whole) brain tissue, normally and when exposed to various amines (usually at a final concentration of 0.12 %), in presence of glucose, sodium lactate, pyruvate, succinate and

Table I. $mm.^3 O_2$ taken up in 2 hours at 37° by 0.5 g. guinea-pig brain tissue, normally, and in the presence of various amines, after 2½ hours' oxidation prior to the addition of glucose, sodium lactate *etc.*

Amine 0.12 %	Metabolite.					
	Saline	Glucose 0.025 %	Sodium lactate 0.025 <i>M</i>	Sodium pyruvate 0.025 <i>M</i>	Sodium succinate 0.05 <i>M</i>	Sodium glutamate 0.05 <i>M</i>
None	287	615	721	785	1698	591
β -Phenylethylamine	303	462	594	520	1739	426
β -Phenyl- β -hydroxy-ethylamine	331	486	576	537	1808	482
β -3:4:5-Trimethoxy-phenylethylamine (Mescaline)	313	392	480	455	1744	436
Tyramine	242	285	296	293	540	275
Indole*	184	376	415	413	1558	221
isoAmylamine	208	287	302	342	1678	298
Neurine	290	672	848	788	1584	573
Cadaverine	317	570	686	613	1780	569
Putrescine	321	601	717	642	1660	540
Ethylamine	369	605	805	—	1585	—
Histamine†	315	670	813	877	1700	652

* 0.5 cc. of a saturated solution of indole in saline at 37° was added to the brain tissue in the saline-phosphate medium, the final volume being 4 cc.

† Present as 0.12 % histamine acid phosphate (neutralised).

glutamate are recorded in Table I. The amounts given are those found in typical experiments and afford a basis for comparison of the inhibitory effects of certain amines on brain oxidations. They are the values obtained after the brain tissue had been allowed largely to deplete itself of oxidisable material by shaking in air at 37° for 2½ hours prior to addition of the metabolites under investigation. The amines had been added to the brain at the commencement of the experiment so that exposure to these substances had occurred for 2½ hours before metabolites were added.

Normally under these experimental conditions constant and reproducible results may be obtained [Quastel and Wheatley, 1932, 1]. The values of the amounts of oxygen taken up by untreated guinea-pig brain in presence of various metabolites are shown on the first line of Table I. The rest of this table shows the effects of exposure of the brain tissue to various amines on the amounts of oxygen consumed. Calculations of the percentage inhibitions effected by these amines on the extra oxygen uptakes due to the addition of metabolites are shown in Table II. Included in Table II, for purposes of comparison, are

Table II. *Percentage inhibition by amines of extra oxygen uptakes due to various metabolites.*

Amine 0.12 %	Glucose	Sodium lactate	Sodium pyruvate	Sodium succinate	Sodium glutamate
β -Phenylethylamine	52	33	44	0	60
β -Phenyl- β -hydroxyethylamine	53	44	54	0	50
Mescaline	76	62	72	0	59
Tyramine	87	88	88	79	89
Indole*	41	47	54	3	87
isoAmylamine	76	79	73	0	70
Neurine	0	0	0	8	7
Cadaverine	26	15	41	0	17
Putrescine	15	9	36	5	28
Histamine	0	0	0	2	0
Narcotic 0.12 %					
Allylisopropylbarbituric acid	73	71	67	2	28
Phenylethylbarbituric acid	94	79	85	0	50
Hyosine	79	73	71	0	60
Chloral	66	90	90	0	62

* See footnote, p. 1610.

the percentage inhibitions of oxygen uptakes effected by typical narcotics—allylisopropyl- and phenylethyl-barbituric acids, hyosine and chloral—the experiments being carried out in the same manner as for the amines.

A characteristic feature of narcotic activity is the large inhibitory effect on the oxidations of glucose, sodium lactate and pyruvate, and little or none, at the concentrations used, on that of sodium succinate. It will be observed from Tables I and II that there exists a similar behaviour on the part of a number of amines.

Whilst cadaverine, putrescine, neurine and ethylamine have relatively little action on the oxidation of glucose or sodium lactate at the concentrations used, tyramine, β -phenylethylamine and β -phenyl- β -hydroxyethylamine have relatively large inhibitory effects. Mescaline (β -3 : 4 : 5-trimethoxyphenylethylamine), well known for its production of visual hallucinations and used of late in psychiatric studies, has similar large effects. Indole¹ and isoamylamine also have considerable inhibitory actions, but histamine is without effect.

¹ Skatole is also highly effective in diminishing the oxidation of glucose by brain.

A notable feature is the exceptionally toxic action of tyramine. Whilst the other derivatives of tyrosine exert specific effects similar to the narcotics, tyramine has a considerable inhibitory action on the oxidation of sodium succinate. This would indicate a more widespread inhibition of the oxidative changes of the brain by tyramine than by the basic amines and narcotics so far investigated. Another point of some interest is the inhibitory action of cadaverine and putrescine on the oxidation of sodium pyruvate with relatively little effect on that of glucose and sodium lactate.

The results as a whole show clearly that amines of the aromatic type, together with *isoamylamine*, exert effects upon brain oxidations which resemble those of the narcotics. There is the same relatively large inhibitory action¹ on the oxidation of glucose and sodium lactate and, except in the case of tyramine, a lack of effect on the oxidation of sodium succinate. The oxidation of sodium glutamate is also considerably affected by the amines and by narcotics. The magnitude of the effects is of the same order as that of the narcotics at the concentrations used.

Whilst the explanation of this behaviour of the amines is probably similar to that of the narcotics, *i.e.* a competition with lactic acid *etc.* for the active surfaces involved, the practical result is of some importance.

Many of the substances studied are normally produced in the bacterial breakdown of amino-acids. Their passage into the liver after absorption into the blood from the intestine is followed by their detoxication—either conjugation as in the case of indole or oxidation as in the case of tyramine. A disturbance in the hepatic detoxicating mechanisms might lead to the circulation in the blood of more than the ordinary quantities of these bases, and this would be expected to lead ultimately to the production of psychological reactions similar to those met in anoxaemia or in the early stages of narcosis. It is not unreasonable therefore to suspect a disturbance in hepatic function as a causative element in certain types of mental disorder. Investigations of the metabolic events in the liver and their possible variations in certain psychotic conditions are clearly necessary and such work is now in progress.

SUMMARY.

1. An investigation has been made of the effects of a number of amines on the oxidations of brain tissue.
2. β -Phenylethylamine, β -phenyl- β -hydroxyethylamine, tyramine, indole, *isoamylamine* and mescaline inhibit strongly the oxidation of glucose, sodium lactate and sodium pyruvate by the brain. They also inhibit the oxidation of sodium glutamate but, with the exception of tyramine, they have little or no effect on the oxidation of sodium succinate. Tyramine presents the only instance so far of an amine or narcotic affecting succinate oxidation at the concentration used. The effects of these amines are similar to, and are of the same order of magnitude as, those of typical narcotics (*e.g.* luminal, hyoscine, chloral).
3. Neurine, cadaverine, putrescine, ethylamine and histamine have relatively little effect on the oxidation by the brain of glucose, sodium lactate or sodium succinate.

¹ The inhibition decreases with decrease in concentration of amine (or of narcotic), definite inhibitions being observed at concentrations considerably lower than 0.12 %. The latter concentration was chosen to give results quite outside experimental error and clearly to indicate the specificity of behaviour.

4. The bearing of these results on the study of certain types of mental disorder is considered.

We are much indebted to the Medical Research Council for a grant to this laboratory in aid of equipment and for a whole time grant to one of us (A. H. M. W.)

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CCXX. STUDIES IN ZYMASIS¹.
IV. THE ACCUMULATION OF ZYMASIC PRODUCTS
IN APPLES DURING SENESCENCE.

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(Received July 26th, 1933.)

ZYMASIS may be measured either in terms of the CO₂ which escapes or in terms of the products which accumulate². In the work described in the present and in the next two papers we have usually employed the "alcohol number" to evaluate zymasis. This number denotes the sum of the concentrations (in g. per unit mass fresh weight of tissue) of ethyl alcohol and of acetaldehyde found in the fruit by analysis at any time.

The apples used throughout this work were stored for us in air for two seasons (1929-30 and 1930-31) at the Low Temperature Research Station, Cambridge, and for one season (1931-32) at the Ditton Laboratory, East Malling, whence they were sent to Newcastle by post³.

In the present paper we report the changes we have found in the alcohol number by analysing apples (a) immediately after their arrival from low temperature stores at different times during the storage season (see Table I and Fig. 1), and (b) after subsequent exposure to air under the experimental conditions employed in the work described in the next two papers⁴ (see Table II and Fig. 2).

This is the first time that data concerning zymasis in air by apples have been collected throughout a storage season.

It had earlier been reported that whereas zymasic products do not accumulate in Newton Wonder apples in early storage [Thomas, 1925], zymasis occurs in several varieties of apples and pears during the oxidase browning that follows injury or physiological disease [Thomas, 1929]. So far as we know, however, no data have been published for older apples before browning begins, although for Bartlett pears it is known that "ripening" is accompanied by a type of zymasis during which acetaldehyde accumulates in relatively high concentrations [Harley and Fisher, 1927].

¹ The following three papers constitute Parts I, II and III of "Studies in Zymasis": Thomas, *Biochem. J.* 1925, **19**, 927; *Ann. Appl. Biol.* 1929, **16**, 444; 1931, **18**, 60.

² By zymasis we mean the process of zymase cleavage of carbohydrates that leads to the production and accumulation of ethyl alcohol and often of acetaldehyde (but in relatively smaller amounts) in plant tissues and the simultaneous production and escape of carbon dioxide.

³ We are indebted to Dr Kidd of Cambridge, and Dr West of East Malling, for arranging that these apples should be stored for us in rooms kept at a constant temperature at these Stations.

⁴ The measurements of the alcohol number which are given in Section III constitute the control figures to the experiments carried out during the storage seasons 1929-30, 1930-31, 1931-32 on the production of ethyl alcohol and acetaldehyde by apples in nitrogen, and in nitrogen-oxygen mixtures. The results of these experiments form the subject matter of papers V and VI of this series.

I. MATERIAL.

Newton Wonder and Bramley's Seedling apples picked from trees in Mr Caleb Lee's orchard at Swanley, Kent, have been used in the major experiments which are described in this and in the two following papers. The apples were stored for us in air at 1° in the season 1929-30, and at 4° in the seasons 1930-31 and 1931-32. Samples have been sent to us by post for these researches¹.

II. METHODS.

(i) *The estimation of the alcohol number.*

The method of estimation of the alcohol number will be reported in a later paper in this series. It is essentially that used by Thomas [1925], but modified so that ethyl alcohol and acetaldehyde may be estimated in the same steam distillate.

In the present paper we generally express our results in terms of the "percentage alcohol number." This means the sum in g. of the masses of ethyl alcohol and of the ethyl alcohol equivalent of acetaldehyde found in 100 g. fresh weight of fruit at the time of the analysis. The data in Table III, show that in these experiments the amount of acetaldehyde which accumulated was usually negligible when compared with that of ethyl alcohol. Hence, in the present work, the alcohol number is virtually a measure of the ethyl alcohol content of the fruit².

(ii) *Experimental procedure.*

When apples were received from cool storage, a sample was immediately analysed to determine the percentage alcohol number, *i.e.* the amount of zymasic products which had accumulated in 100 g. fresh weight of the fruit during storage at 1° or 3-4°, *plus* a day in the post. This number will be termed the percentage alcohol number of the "storage controls."

The remaining apples were then used to determine the percentage alcohol numbers of what we shall term in this present paper and in Part VI of this series the "conditions controls." To obtain this number, the apples, before they were analysed, were exposed for a period to the conditions stated below.

The sample of fruit was weighed and placed under a glass hemisphere with a wide equatorial flange, which was then fixed to a ground glass plate by means of paraffin wax. Through the pole of the container passed inlet and outlet tubes for the air stream. The fruit chamber was secured to an iron frame and immersed in a water-bath, the temperature of which was kept within 0.2° of 23° by means of a thermostat. CO₂-free air was drawn through the chamber at the rate of 2 litres per hour for a period of 120 to 140 hours. The issuing gas was bubbled through 50 cc. of concentrated sulphuric acid in a wash-bottle to trap any transpired alcohol. At the end of the period of exposure to the higher temperature, the fruit was re-weighed and analysed. The acid from the wash-bottle was added to aqueous potassium dichromate, and when oxidation was complete the mixture was distilled. The amount of alcohol transpired was calculated from the acetic acid value of this distillate. In Table II allowances have been made for this transpired alcohol.

¹ We are deeply indebted to Dr West for arranging to secure these varieties from a single orchard, and for sending samples from the air stores at the times we wanted them for our experiments.

² This is not always the case. Thus, for example, after CO₂-zymesis [Thomas, 1925], the ratio ethyl alcohol/acetaldehyde is sometimes less than 2/1. The alcohol number would then be over 50 % greater than the ethyl alcohol content of the fruit.

III. RESULTS.

The results for the storage controls are given in Table I and Fig. 1. Table II and Fig. 2 deal similarly with the conditions controls. In Table III are given the amounts of acetaldehyde and ethyl alcohol found in apples at different times during the season 1931-32. The figures in this table have been extracted from the figures for the percentage alcohol number given in Tables I and II.

Table I (see Fig. 1). *Percentage concentration of products of zymasis in air-stored apples (storage controls).*

Variety of fruit	Date	Days in store	Percentage alcohol number	Remarks (state of fruit, etc.)
Newton Wonder:				
Season 1929-30	21. iv. 30	195	0.008	Sound
	16. v. 30	225		
	5. vi. 30	250	0.030	Superficial scald
	12. vi. 30	255	0.025	"
	19. vi. 30	265	0.030	"
	25. vi. 30	270	0.025	"
Season 1930-31	17. vii. 30	From orchard	0.004	Very young. Green. Av. wt. 12 g.
	25. ix. 30	—	0.003	Young. Green. Av. wt. 120 g.
	20. x. 30	27	0.005	Green-yellow. Sound
	9. i. 31	105	0.010	Yellow-green. Wilted
	15. i. 31	112	0.011	"
	23. i. 31	120	0.020	"
	1. vii. 31	From orchard	0.004	Very young. Green. Av. wt. 8.5 g.
Season 1931-32	18. vii. 31	"	0.004	" 20.0 g.
	30. viii. 31	"	0.004	" 70.0 g.
	28. x. 31	28	0.005	Hard. Green
	9. xii. 31	70	0.003	Green to yellow-green
	6. i. 32	97	0.007	Yellow. Internal breakdown
	27. i. 32	119	0.019	Green-yellow. First sign of wrinkling
	25. ii. 32	147	0.018	Yellow. Wilted
Bramley's Seedling:				
Season 1930-31	13. xi. 30	50	0.015	Hard. Green
	20. xi. 30	55	0.010	
	7. i. 31	105	0.015	Green
	21. i. 31	120	0.030	Green. Wilted. Keeping badly, soft spots on fruit
	28. i. 31	127	0.033	Green-yellow. Slightly wilted
	6. ii. 31	135	0.025	
	11. ii. 31	142	0.030	Green-yellow. " Badly wilted
	4. iii. 31	165	0.050	Badly wilted and scalded
Season 1931-32	1. vii. 31	From orchard	0.003	Small. Green. Av. wt. 23 g.
	18. vii. 31	"	0.004	" 40 g.
	30. viii. 31	"	0.004	Young. Green. Av. wt. 125 g.
	10. xi. 31	40	0.018	Green
	6. i. 32	98	0.014	"
	16. iii. 32	170	0.025	Green-yellow
	1. iv. 32	185	0.045	Green-yellow. First sign of wilting

IV. CONCLUSIONS.

(i) *The increase in ageing apples of the alcohol number of storage controls.* The data reported in Table I, and charted in Fig. 1 confirm the earlier observations of Thomas [1925] that the concentration of zymasic products in Newton Wonder apples early in the storage period does not rise above 0.005 % in cool storage at 1-4°. The alcohol numbers for the storage controls of Bramley's Seedling apples during this same period were usually higher than those for

Table II (see Fig. 2). *Increase in the percentage alcohol number in apples in air at 23° in the respiration chamber (conditions controls).*

No. of exp.	State of fruit		Date exp. begins	Duration of exp. (hrs.)	No. of apples in sample	Wt. of sample		Percentage alcohol number after 100 hrs. exposure to air at 23° following low temperature storage (allowing for transpired alcohol)	Increase in percentage alcohol number during 100 hrs. exposure to air at 23°	
						Initial g.	Final g.			
1	2		3	4	5	6	7	8	9	
Newton Wonder.										
Season 1930-31:										
7	Wilted.	Superficial scald	11. vi. 30	114½	1	—	72.0	0.150	0.120	
Season 1930-31:										
20	Green.	Hard. Young	1. x. 30	142	1	—	100.5	0.017	0.014	
A	Yellow-green,	wilted	9. i. 31	117½	1	—	133.5	0.025	0.015	
B	Yellow,	wilted	16. i. 31	121½	1	—	97.5	0.150	0.140	
Season 1931-32:										
76	Green.	Hard. Very young	8. vii. 31	116¼	12	67.0	60.0	0.024	0.02	
C	Green "		17. vii. 31	113	6	134.5	132.5	0.019	0.014	
84			26. viii. 31	122	3	212.0	201.0	0.019	0.015	
98			28. x. 31	140	2	321.0	312.5	0.011	0.007	
112			Green to yellow-green	10. xii. 31	141½	2	279.0	270.0	0.013	0.010
128	Green-yellow; first sign of wrinkling		27. i. 32	140½	1	97.5	97.0	0.034	0.015	
H	"		10. ii. 32	119½	1	—	—	0.077	0.060	
133			24. ii. 32	138	1	87.0	83.5	0.040	0.022	
Bramley's Seedling.										
Season 1930-31:										
D	Green.	Hard	20. xi. 30	118½	1	141.0	138.0	0.022	0.013	
E	Yellow-green,	slightly wilted	23. i. 31	117	1	—	125.5	0.090	0.060	
46	"		29. i. 31	116	1	—	170.0	0.093	0.060	
47			29. i. 31	116	1	—	130.0	0.100	0.060	
48			Green-yellow	30. i. 31	116½	1	—	177.0	0.076	0.043
49			Yellow-green	30. i. 31	116½	1	—	160.0	0.073	0.037
55	Yellow.	Badly scalded	5. iii. 31	116	1	—	147.0	0.200	0.150	
56	"		6. iii. 31	118	1	—	233.0	0.220	0.170	
Season 1931-32:										
75	Green.	Hard. Very young	8. vii. 31	116¼	8	172.0	161.5	0.010	0.007	
F	"		17. vii. 31	113	5	192.5	189.5	0.010	0.007	
88			2. ix. 31	140½	2	218.5	218.0	0.016	0.012	
105			Green	11. xi. 31	115½	1	207.5	202.0	0.070	0.052
120			"	6. i. 32	138½	1	158.0	157.5	0.074	0.060
137	Green-yellow		16. iii. 32	140	1	167.5	164.0	0.110	0.090	
141	Green-yellow; first sign of wilting		31. iii. 32	116½	1	127.5	126.0	0.100	0.050	

Up to May 1931, the weight used in arriving at the percentage concentration of products of zymasis was the final weight of the apple. After this it was realised that the use of the final weight was open to objection, and since then the initial weight has been used. The loss of weight is never more than 6% in a sound apple and is usually of the order of 1 to 3% and, as all the figures in any series are calculated to the same basis, the error is not serious.

The original experimental numbers are retained for the convenience of such readers as may have access to Fidler's Ph.D. thesis [1932].

The percentage alcohol numbers given in column 9 represent the difference between column 8 and the corresponding percentage alcohol numbers in Table I. They constitute the conditions control values which were used as a basis for measuring zymasis owing to oxygen shortage in the work which is described later [Thomas and Fidler, 1933, Section II].

Newton Wonder apples, but did not significantly exceed 0.015%. We infer that the rate of production of ethyl alcohol in storage controls during the earlier phase of storage is very slow. If zymasis and cell oxidations are linked processes, this slow rate may be due to the vigour with which some precursor of ethyl alcohol is oxidised during this phase.

Table III. *Production of acetaldehyde and ethyl alcohol in air by Newton Wonder and Bramley's Seedling apples during 1931-32.*

Time of analysis	Variety of apple	Number of exp.	Date	g. per 100 g. apple	
				Acet-aldehyde	Ethyl alcohol
When received from store	Newton Wonder	—	1. vii. 31	0.0005	0.003
		—	18. vii. 31	0.0006	0.003
		—	30. viii. 31	0.0005	0.004
		—	28. x. 31	0.0003	0.004
		—	9. xii. 31	0.0004	0.003
		—	6. i. 32	0.0005	0.007
	Bramley's Seedling	—	27. i. 32	0.0008	0.018
		—	25. ii. 32	0.0028	0.015
		—	1. vii. 31	0.0002	0.003
		—	18. vii. 31	0.0005	0.003
		—	30. viii. 31	0.0010	0.003
		—	10. xi. 31	0.0009	0.017
	Newton Wonder	—	6. i. 32	Nil	0.014
		—	16. iii. 32	0.0019	0.024
		—	1. iv. 32	0.0026	0.042
Following 100 hours' exposure to air at 23°	Newton Wonder	76	8. vii. 31	0.0018	0.022
		84	26. viii. 31	Nil	0.019
		98	28. x. 31	0.0007	0.011
		112	10. xii. 31	0.0006	0.013
		128	27. i. 32	0.0036	0.030
		H	10. ii. 32	0.0032	0.074
	Bramley's Seedling	75	8. vii. 31	0.0005	0.009
		88	2. ix. 31	0.0006	0.015
		105	11. xi. 31	0.0024	0.067
		120	6. i. 32	0.0033	0.071
		137	16. iii. 32	0.0043	0.108
		141	31. iii. 32	0.0047	0.090

The change in colour of an apple to a yellow-green, and, at a later date, the incidence of severe wilting and superficial scald, which occurs when the apple is full yellow, are the only external indications of the physiological state of the fruit. In wilted apples the skin can readily be pushed into ridges and later becomes noticeably wrinkled. Superficial scald means that there are dry brown patches just under the skin: this condition may be succeeded by deep scald, in which the whole of the skin is involved, and the browning extends deeply into the flesh of the fruit.

It will be seen from Fig. 1 that as Newton Wonder and Bramley's Seedling apples change in colour from green to green-yellow, the alcohol numbers of the apples rise, and as the apples become more yellow and wilted, the graph of the alcohol number takes on a steep upward path¹. The data of Table III indicate that this rise in the alcohol number is compounded of a rise in both the ethyl alcohol and acetaldehyde contents of the apples. The highest recorded values for the percentage alcohol numbers in apples which show no browning, from an air store, are 0.03 in Newton Wonder apples in June 1930, and 0.05 in Bramley's Seedling apples in March 1931.

¹ A few experiments permit us to compare the Cleopatra apple, a variety shipped from overseas, with these two varieties of home-grown apples. Thus whereas the percentage alcohol number of green-yellow Cleopatras was less than 0.007, this number had increased to 0.023 by the time this variety had turned yellow. We note that the alcohol number of apples showing no browning from ships' holds which have been well ventilated may indicate the occurrence of recent ymasis. But there is no evidence yet of the alcohol number in apples from well ventilated air stores reaching the magnitudes found in the flesh tissue after faulty ventilation [Thomas, 1931].

It appears, therefore, that the rate of zymasis after a slow initial period of varying extent becomes increasingly accelerated as the apples age further. Possibly there is always a critical phase in the storage life of the fruit when certain changes become autocatalytic. Thus in a rough grouping of Newton Wonder apples we might place in one class those apples in which the percentage alcohol number is not greater than 0.005, and in the other those apples in which the alcohol number is steadily increasing beyond this figure. Similarly, for Bramley's Seedling apples, the line of demarcation may be placed at 0.015. In our view apples in the second class (*i.e.* those which have reached the critical phase) cannot be regarded as physiologically healthy, for, in our experience, when zymasis becomes relatively rapid the browning of the flesh tissue is not far distant.

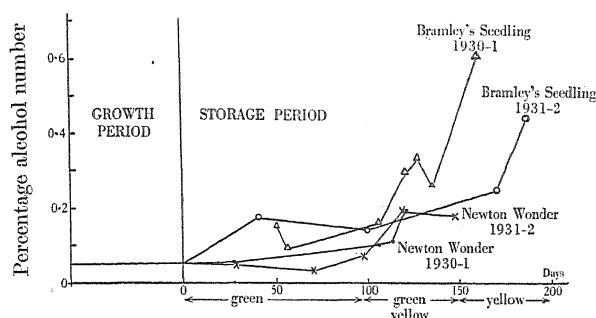


Fig. 1. Increase in percentage alcohol number in apples during storage.

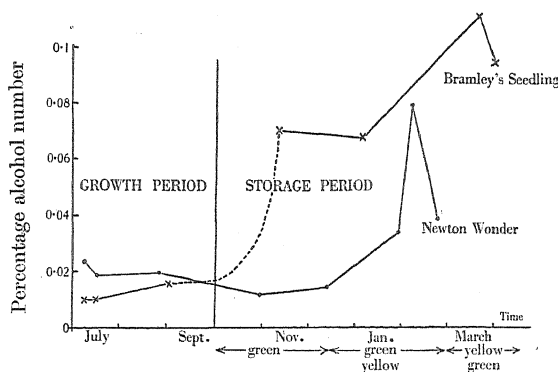


Fig. 2. Percentage alcohol number in apples after 100 hours' exposure to air at 23°, following (a) removal from the tree during the growing period, or (b) low temperature storage (1930-31)¹.

(ii) *The increase in ageing apples of the alcohol number of conditions controls.* The data in column 9 in Table II and the differences in the heights above the *x*-axis of comparable points in Figs. 1 and 2, show that the effect of placing

¹ We have never observed the alcohol number of an apple to rise until some months after it is removed from the tree. Accordingly, part of the graph for Bramley's Seedling apples during 1931-32 has been drawn with a broken line at a level which we consider approximately to represent the true state of affairs.

Newton Wonder and Bramley's Seedling apples¹ in a closed space in moving air at 23° at any time in the storage season is to increase the alcohol number². Thus, for example, except in young Bramley's Seedling apples, the alcohol number for apples after 5 days' exposure to air, under experimental conditions at 23°, is higher than in apples received from a 4° store 1 to 2 months later. The cause of this may only be suggested. The two factors that, either together or singly, may influence zymasis are humidity and temperature. Of the effect of humidity as a single factor, nothing is known. It may well be that the question is related to temperature alone. Although the temperature of the experiment is 20° above that of the store, yet it is only 8° above the average temperature of the laboratory, so that the oxygen content of the intercellular spaces of the fruit should still be adequate for aerobic respiration.

It is well known that increase in temperature accelerates the rate of senescence in apples. Possibly one of the features of senescence is the progressive retardation of oxidative processes, and this may be the factor which leads to the observed increase in the alcohol number.

Changes in the alcohol number of the conditions controls at different periods in the storage season point to the same conclusions that we reached from the data given in the last sub-section. Thus the form of the graphs for the conditions controls is similar to that of the graphs in Fig. 1 for the storage controls, the rise in the alcohol number occurring at the same state of the fruit³. Once more the results (see Table II) may be divided into two groups. Before a certain critical phase the alcohol number of a conditions control, although higher than that of a storage control, remains relatively low. Later, when senescent characters are seen the disturbance in metabolism leading to zymasis becomes increasingly marked, as is evidenced by the rapid increase in the alcohol number of the conditions controls.

SUMMARY.

1. Little zymasis occurs in Newton Wonder and Bramley's Seedling apples at the beginning of the storage season and up to the beginning of the change from green to yellow-green, when these varieties are stored in air at 1-4°.
2. Senescence is accompanied by a change in the respiratory metabolism of the cells of the fruit, leading to the accumulation in an apple stored in air of considerable amounts of ethyl alcohol and smaller amounts of acetaldehyde, *i.e.* the alcohol number of the apple rises.
3. Increase of temperature, which is known to accelerate the rate of senescence in an apple, leads to an increase in the alcohol number of the apple.

The work described in this, and in the following paper, was carried out in the Botany Department, Armstrong College, under the supervision of

¹ The Cleopatra apple responded similarly to this transfer to experimental conditions, thus in green to green-yellow apples the percentage alcohol number increased by 0.005 during 100 hours' exposure to air under experimental conditions at 23°.

² Hence, to eliminate the effect of experimental conditions as such, conditions controls and not storage controls should always be used in quantitative researches in which zymasis is induced by shortage of oxygen or by the presence of foreign substances [see *e.g.* Thomas and Fidler, 1933].

³ It is not claimed that zymasis in air at 23° may be calculated from Fig. 2. Regarding the increased zymasis in air, there are two alternatives: (*a*) that the increased rate may be maintained over long periods, and (*b*) that the increase may take place only at the beginning of the exposure to the new experimental conditions. The present research is not concerned with the elucidation of these questions.

Mr Meirion Thomas, to whom I am indebted for continued advice and helpful criticism. My thanks are due to Armstrong College for a College Research Studentship during 1929-30, and to the Department of Scientific and Industrial Research for maintenance grants during 1929-30, and 1930-31, and for payment as a Research Assistant during 1931-32.

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CCXXI. STUDIES IN ZYMASIS.

V. SEASONAL FLUCTUATIONS IN ZYMASIS AND IN CARBON DIOXIDE/ALCOHOL NUMBER RATIOS IN APPLES IN THE ABSENCE OF OXYGEN.

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(Received July 26th, 1933.)

TOWARDS the end of the nineteenth century it was realised that the cells of many higher plants could form ethyl alcohol in the absence of oxygen [see literature quoted by Kostytschew, 1927], and several workers made simultaneous measurements of the production of ethyl alcohol and CO_2 in anaerobiosis.

Recent comparisons of anaerobic and aerobic metabolism have led to an alteration of biological outlook on cell respiration in animals, yeast and bacteria. It was shown by Blackman [1928] that the cells of higher plants fall into line with those of other organisms. Blackman based his conclusions on measurements of the CO_2 output of stored Bramley's Seedling apples in air and in nitrogen. At that time he made the primary assumption in his paper that the ratio CO_2 /ethyl alcohol in the anaerobic respiration of this variety is unity. For example, he wrote, "in nitrogen, Group D (by which he implies intermediate compounds between hexoses and ethyl alcohol) proceed quantitatively to the two final products CO_2 and alcohol in the usual ratio," and, "it will be held that for one atom of carbon thus detected (as CO_2) there are two atoms of carbon excreted into the tissues as alcohol." Actually, as will be pointed out later, the precise value of the ratio CO_2 /ethyl alcohol, although affecting his arithmetical deductions slightly, does not influence his main argument. Some of his conclusions, however, are only valid provided the ratio, whatever may be its magnitude, keeps constant throughout the storage season. It was the aim of the present investigation to test whether this was the case. The quantitative methods of estimating ethyl alcohol and acetaldehyde, which will be described in a later paper, have been employed to determine the CO_2 /alcohol number [see Fidler, 1933] ratio in selected varieties of apples placed under anaerobic conditions at different times during the storage season. The results for Bramley's Seedling and Newton Wonder apples are given in Section I, and the results of preliminary measurements of the CO_2 /alcohol number ratio in several other varieties of apples are shown in an appendix.

MATERIALS AND METHODS.

The apples used were from the population used throughout this work [Fidler, 1933]. They were placed in the apparatus already described [Fidler, 1933] at a temperature of 23° . The gas stream, after passing through the fruit chamber, bubbled through a wash-bottle containing sulphuric acid (as described in the first paper), and then through a Pettenkofer tube containing 60 cc. of standard baryta. These tubes were changed at intervals.

After the apples had attained the temperature of the bath, air was drawn through at a rapid rate for a period varying between 18 and 24 hours. The air-line thus being fixed, the air current was changed for pure nitrogen at the standard rate of 2 litres per hour¹.

The nitrogen used in the experiments was commercial cylinder nitrogen, and was found to contain between 0.5 and 1.2 % oxygen. This was removed by passing the gas over copper which was heated electrically to 650–700°. Gas analyses of the purified gas showed that this treatment reduced the percentage of oxygen to less than 0.05.

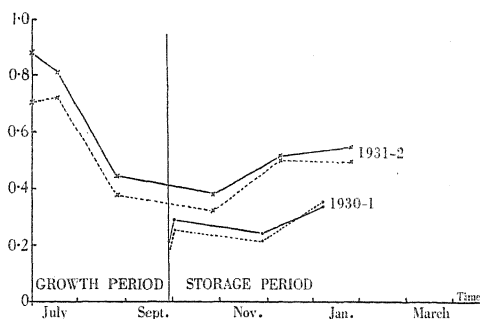


Fig. 1. Rates of CO₂-production (—), and rates of increase in the alcohol number (....) in Newton Wonder apples in nitrogen at 23°. Ordinate: g. CO₂ produced by 100 g. fruit during 100 hours or increase in alcohol number during 100 hours

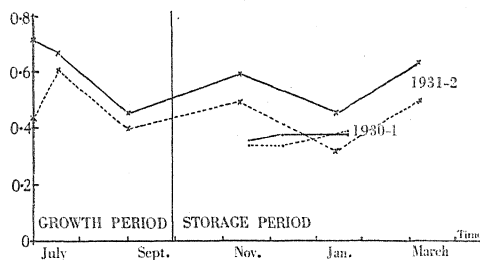


Fig. 2. Rates of CO₂-production (—), and rates of increase in the alcohol number (....) in Bramley's Seedling apples in nitrogen at 23°. Ordinate: g. CO₂ produced by 100 g. fruit during 100 hours or increase in alcohol number during 100 hours.

At the end of the exposure to the gas (4 to 5 days), the fruit was removed from the apparatus, weighed, and the alcohol number was estimated. The CO₂ absorbed by the baryta was estimated by titrating the barium hydroxide-carbonate mixture against standard hydrochloric acid using phenolphthalein as an indicator.

Part of the same sample of fruit was analysed immediately on being received, and the alcohol number thus obtained was subtracted from that found in the above experiment, in order to get a measure of the effect of the absence of oxygen on zymasis.

¹ The rate of passage of the gas stream was measured in the first instance by collecting the emergent gas in a graduated jar over water. The rate of bubbling through a wash-bottle containing KOH (to remove CO₂ from gas entering the apparatus) was then noted. This rate of bubbling was reproduced in subsequent experiments.

Table I. CO_2 /alcohol number in apples in pure nitrogen at 23°.
(See Figs. 1 and 2.)

No. of exp.	Variety of fruit	State of fruit	Date exp. begins	Duration of exposure to nitrogen (hrs.)	No. of apples in sample	Wt. of sample (g.)	Percentage CO_2 production (g./100 hrs.)	Increase in percentage alcohol number during 100 hrs. exposure to nitrogen, allowing for transpired alcohol	Ratio CO_2 /alcohol number (both expressed as gram-molecular equivalents)*	Glycolysis in C units/ CO_2 in C unit†
1	2	3	4	5	6	Initial	Final	10	11	12
Season 1929-30:										
Not plotted	11 Newton	Sound	I. iv. 30	121½	1	—	99.5	0.37	100:86	2.7
	13 Wonder	Superficial scald	14. v. 30	123½	1	—	88.5	0.40	100:91	2.8
Season 1930-31:										
17 Newton	Green. Hard		24. ix. 30	97½	1	—	112.5	0.19	100:89	2.8
21 Newton	Young. Hard		1. x. 30	100½	1	—	144.0	0.25	100:84	2.7
30 Wonder	Green to green-yellow		27. xi. 30	97	1	—	193.0	0.21	100:84	2.7
37	Yellow, slightly wilted		8. i. 31	99½	1	—	105.0	0.35	100:99	3.0
Season 1931-32:										
73 Newton	Green. Hard. Very young		1. vii. 31	97½	12	103.0	96.5	0.70	100:75	2.5
79 Wonder	"		17. vii. 31	95	9	174.5	167.0	0.73	100:86	2.7
81	"		26. viii. 31	98½	3	208.0	201.0	0.38	100:81	2.6
95	Green		28. x. 31	99	2	252.5	246.0	0.32	100:80	2.6
109	Green to yellow-green		10. xii. 31	98½	2	298.5	293.5	0.50	100:93	2.8
125	Green-yellow. First sign of wilting		27. i. 32	99	1	84.0	81.5	0.50	100:88	2.7

Season 1932-33:

143	Newton Wonder	Small and young	16. viii. 32	120	4	276.0	273.5	0.264	0.19	100:69	2.4
Not plotted	I { a b c	Green	19. x. 32	119	2	260.0	254.5	0.254	0.15	100:56	2.1
		"	19. x. 32	119	2	306.0	301.5	0.276	0.18	100:60	2.2
		"	19. x. 32	119	2	296.0	291.5	0.316	0.20	100:66	2.4
		"	10. xi. 32	95½	2	226.5	225.5	0.343	0.24	100:68	2.4
		"	10. xi. 32	95½	2	226.0	222.0	0.315	0.24	100:74	2.5
III { a b c	"	10. xi. 32	95½	2	235.0	230.5	0.329	0.24	100:70	2.4	

Season 1930-31:

25	Branley's	Green. Hard. Young	19. xi. 30	97½	1	—	148.5	0.356	0.34	100:91	2.8
34	Seedling	Green. Firm	10. xii. 30	97	1	—	124.5	0.377	0.31	100:88	2.7
43		Green to green-yellow.	22. i. 31	98½	1	—	134.5	0.379	0.39	100:97	3.0
		Slight wilting and superficial scald									

Season 1931-32:

72	Branley's	Green. Hard. Very young	1. vii. 31	97½	11	238.5	233.0	0.714	0.43	100:58	2.2
78	Seedling	"	17. vii. 31	95	5	200.0	192.0	0.667	0.60	100:93	2.7
85		"	2. ix. 31	99	2	272.5	271.5	0.455	0.40	100:83	2.6
102		Very green	11. xi. 31	98½	1	223.0	218.0	0.592	0.50	100:80	2.6
117		Green	12. i. 32	99	1	187.5	185.5	0.456	0.32	100:67	2.4
134		Green-yellow	6. iii. 32	97½	1	138.0	134.5	0.631	0.50	100:76	2.6

Season 1932-33:

142	Branley's	Small and young	16. viii. 32	120	4	247.0	247.0	0.252	0.25	100:91	2.9
Not plotted	III { a b c	Seedling									
		Branley's	24. xi. 32	95½	2	230.5	257.5	0.408	0.36	100:85	2.7
		Seedling	24. xi. 32	95½	2	265.0	260.5	0.362	0.33	100:86	2.7
		"	24. xi. 32	95½	2	262.5	257.5	0.396	0.36	100:92	2.8

* The ratios given in column 11 are for the percentage CO₂ produced in unit time, and expressed as molecular equivalents/the percentage increase in the alcohol number in unit time, also expressed as molecular equivalents. It is of interest to note that the ratio ethyl alcohol/acetaldelyde was never less than 75/1. Since the present research is concerned only with the alcohol number and was not undertaken to determine this ratio, the alcohol numbers in the tables are not divided between ethyl alcohol and acetaldelyde.

+ Blackman [1928] prefers to summarise the reaction as a quotient, glycolysis in carbon units/N.R. in carbon units. A column has been added (12) to permit of comparison with his results, should this be desired later. Each figure in this column represents the sum of the carbon equivalents of CO₂ (column 9) and of ethyl alcohol (column 10) divided by the carbon equivalent of CO₂ (column 9). No further mention will be made of this column here.

RESULTS AND CONCLUSIONS.

From the data for the alcohol number in column 10 of Table I, and the graphs of these data in Figs. 1 and 2, we see that in both Bramley's Seedling and Newton Wonder apples the average rate of anaerobic zymasis over a period of 100 hours appears to fall sharply from the value for samples of growing apples in late July to that for fully grown green apples. These changes for growing apples will be further investigated in the future. Our results for fully grown stored apples suggest that during the early stage of storage the rate of anaerobic zymasis changes but little. When, however, the colour change from green to yellow sets in, the rate of anaerobic zymasis appears to be accelerated.

Apparently the CO_2 output of these two varieties under anaerobic conditions fluctuates seasonally in a similar fashion (column 9, Table I, and Figs. 1 and 2). Thus the average rate of CO_2 output during a period of 100 hours of anaerobiosis falls sharply during the later phases of growth to a value which does not change significantly during the early phases of storage. The graphs suggest that the rate of CO_2 output by apples under anaerobic conditions increases as they turn yellow. If a connection is assumed to exist between anaerobic and aerobic processes, this rise, and also the fall during late orchard life, is what would be expected from the published results of other workers for the CO_2 output of apples in air [see Hardy, 1933].

The most important deduction to be made from the present data is that the ratio CO_2 /alcohol number (Table I, column 11) does not alter significantly with the season in spite of these seasonal changes in the rates of anaerobic zymasis (Table I, column 10), and anaerobic CO_2 output (Table I, column 9). It appears, therefore, that these two magnitudes fluctuate proportionally, and this conclusion is borne out by the parallelism of the courses followed by the graphs for the alcohol number and CO_2 -output in Figs. 1 and 2.

Table II. *Mean values for CO_2 /alcohol number in stored Newton Wonder and Bramley's Seedling apples.*

Variety	Season	Mean value of CO_2 /alcohol number	Deviation from mean	No. of measurements from which mean value is calculated
Newton Wonder	1929-30	100:88.5	± 2.5	2
	1930-31	100:89	$\begin{cases} +10 \\ -5 \end{cases}$	4
	1931-32	100:87	± 6	3
	1932-33	100:65.5	$\begin{cases} +8 \\ -5 \end{cases}$	6
	1929-30-31-32-33*	100:72.5	$\begin{cases} +27 \\ -16 \end{cases}$	15
Bramley's Seedling	1930-31	100:92	± 5	3
	1931-32	100:72	$\begin{cases} +8 \\ -5 \end{cases}$	3
	1932-33	100:87.5	$\begin{cases} +4.5 \\ -2.5 \end{cases}$	3
	1930-31-32-33*	100:84.5	$\begin{cases} +13 \\ -17 \end{cases}$	9

* See Introduction, and also Thomas and Fidler [1933].

This substantiates the important assumption implicit in Blackman's paper that the CO_2 /alcohol ratio remained constant during the period of storage in which his experiments were carried out. Considerable interest centres, therefore, in the mean value of this ratio for a given season.

The data in the above table show that, except in Newton Wonder apples in seasons 1929-32, there is a different mean value for the CO_2 /alcohol number ratio in each season. The difference in the mean value of this ratio for different seasons may be accidental, but experiments with large samples would be needed to settle this point.

From two observations on leaves of *Acer platanoides*, Kostytschew [1913] inferred that the ratio CO_2 /ethyl alcohol fluctuates with the stage of development of the tissue. But in the present experiments the fluctuations, though considerable, were not regular. They might be attributed to a combination of experimental error, the occurrence of physiological races within the varieties, and the use of small samples. Probably the last is the most important cause: for, as will be shown in a later paper, this sampling error is much larger than the maximum error encountered in the estimation of ethyl alcohol and acetaldehyde in apples.

The average value for the CO_2 /alcohol number ratio in Bramley's Seedling and Newton Wonder apples may be placed at 100/80-85. We have, however, obtained some evidence (see Appendix) of varietal differences in regard to the ratios.

As the CO_2 /alcohol number ratio in molecular equivalents is not unity, as demanded by the usual equation for alcoholic fermentation, $\text{C}_6\text{H}_{12}\text{O}_6 = 2\text{CO}_2 + 2\text{C}_2\text{H}_5\text{O}$, Blackman's method of evaluating glycolysis must be slightly modified. He assumes that the ratio is unity, and he calculates glycolysis from CO_2 output in nitrogen by multiplying this figure by 3. If, however, the CO_2 /alcohol number ratio is 100/85, only 1.7 atomic proportions of carbon would be lost as ethyl alcohol for each one lost as CO_2 , so that glycolysis would be 2.7 times the CO_2 output in nitrogen. In view of the fluctuations about a mean value of 100/85, the CO_2 /alcohol number ratio should be measured in a sample of the same fruit before attempting to estimate glycolysis. Many other examples are known of CO_2 /ethyl alcohol ratios in which CO_2 predominates, and only rarely has a ratio of unity been reported and confirmed. Thus Boysen-Jensen [1922] for grapes and carrots and Kostytschew [1913] for many tissues including apples obtained ratios ranging from 100/40 to 100/80. Both conclude that anaerobic respiration in higher plants is a mixed reaction which consists only in part of alcoholic fermentation, the accompanying reaction being independent of glycolysis.

This latter conclusion cannot be accepted unconditionally, for products of zymasic cleavage not estimated by the present method may account for the low ratios obtained. Acetaldehyde, however, cannot be one of these, since this compound is included in the alcohol number.

SUMMARY.

1. Zymasis in nitrogen follows the same seasonal curve as CO_2 output in air (O.R.).
2. In any one season, the ratio CO_2 /alcohol number (which is practically identical with the ratio CO_2 /ethyl alcohol) fluctuates widely about a mean value, but this mean does not alter as apples age.
3. The mean value for this ratio in Newton Wonder and Bramley's Seedling apples during three seasons is of the order 100/80-85.

4. The mean value for the CO_2 /alcohol number ratio may vary from season to season.

5. The results of preliminary experiments suggest that there are great varietal differences in the CO_2 /alcohol number ratio; this ratio fluctuates between 100/47 to 100/92 in small samples of different varieties of ripe apples.

In addition to the acknowledgments which have already been made [Fidler, 1933], I wish to thank the Armstrong College Research Committee for a grant for the purchase of nitrogen.

APPENDIX.

A SURVEY OF CO_2 /ALCOHOL NUMBER IN SEVERAL VARIETIES OF APPLES.

The following is an account of a short survey of CO_2 /alcohol number in several varieties of ripe apples purchased from local fruiterers. Estimations were carried out precisely as described in the main paper.

Variety	Country of origin	Date of commencement of exp.	Duration of exposure to nitrogen (hours)	g. CO_2 per 100 g. fruit per 100 hours	Increase in the % alcohol number in 100 hours (corrected for control values)	CO_2 /alcohol number
Dougherty	Australia	23. viii. 32	120	0.169	0.14	100:81
Gravenstein	U.S.A.	23. viii. 32	120	0.277	0.19	100:65
Sturmer	Australia	23. viii. 32	120	0.327	0.26	100:77
Keswick	England	1. ix. 32	120	0.264	0.16	100:57
Beauty of Bath	England	1. ix. 32	120	0.209	0.13	100:61
Belflower	France	1. ix. 32	120	0.212	0.15	100:68
Maiden's Blush	U.S.A.	28. ix. 32	119 $\frac{1}{2}$	0.289	0.22	100:73
Wealthy	U.S.A.	28. ix. 32	119 $\frac{1}{2}$	0.167	0.08	100:47
Jonathan	U.S.A.	11. x. 32	120 $\frac{1}{2}$	0.151	0.08	100:54
McIntosh Red	Canada	11. x. 32	120 $\frac{1}{2}$	0.193	0.17	100:85
Cleopatra	Tasmania	11. vii. 30	99 $\frac{1}{2}$	0.164	0.16	100:93
Cleopatra	Tasmania	6. v. 31	98 $\frac{3}{4}$	0.255	0.21	100:85
Newtown Pippin	U.S.A.	8. iii. 33	114 $\frac{1}{2}$	0.113	0.10	100:90
Newtown Pippin	U.S.A.	25. iv. 33	148 $\frac{3}{4}$	0.177	0.15	100:81
Granny Smith	Australia	9. vi. 33	49	0.278	0.20	100:65

It appears that as for Bramley's Seedling and Newton Wonder apples, so also for these other varieties, the ratio CO_2 /alcohol number is always higher than that required by the usual equation for alcoholic fermentation. As small samples were used, the ratios given in the table cannot be taken as true means, but the differences shown suggest that the mean ratios may possibly vary greatly in different varieties of apples.

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CCXXII. STUDIES IN ZYMASIS.

VI. ZYMASIS BY APPLES IN RELATION TO OXYGEN CONCENTRATION.

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(Received July 26th, 1933.)

THE ensuing subject matter deals principally with the problem of determining the concentration of oxygen at which the anaerobic respiration of apples becomes extinguished. Pure nitrogen has been used in most of the recent work on the anaerobic respiration of plants, and we propose to follow Blackman [1928] and Parija [1928] in describing the CO_2 component of anaerobic respiration as nitrogen respiration (N.R.). We shall refer to the concentration of oxygen that extinguishes N.R. (*i.e.* anaerobic respiration) as the extinction point of N.R.

Bramley's Seedling apple was selected as a subject for experiment because much important work has been performed on this variety at the Botany School and at the Low Temperature Station at Cambridge; but experiments have also been performed with the Newton Wonder apple.

The scope of this research was widened to test Blackman's contention (see below) that, even for a single variety for a given season, the extinction point of N.R. is not a fixed magnitude throughout the storage period but shifts to higher values as the fruit ages. The extinction points of N.R. for Newton Wonder and Bramley's Seedling apples have therefore been determined late as well as early in the storage season. Further, as our experiments extended over 2 years, we can consider whether seasonal factors affect the time at which the shift of the extinction point begins in stored fruit.

The novelty of our work resides essentially in the method adopted for finding the extinction point for N.R. The method is based on the facts, established by earlier quantitative researches [Thomas, 1925], first that healthy apples do not normally undergo zymasis in air, and secondly that under anaerobic conditions ethyl alcohol accumulates in the flesh tissue until this is dead. It is argued that zymasis probably also occurs in low concentrations of oxygen, and that there is for any apple in a given physiological state a limiting concentration of oxygen that extinguishes this process.

By our methods of analysis, ethyl alcohol, acetaldehyde (which also accumulates in small amounts) and possibly traces of other compounds which are volatile in steam, are measured together to give what we term the "alcohol number" [see Fidler, 1933, 1]. This number may be taken as a measure of the zymasic products which have accumulated in the flesh tissue. Under anaerobic conditions CO_2 (Blackman's N.R.) is simultaneously produced, and there is evidence for Bramley's Seedling and Newton Wonder apples that the greater part of this N.R. is due to zymasis, and that the ratio alcohol number/N.R. for a given variety is constant during a single season [Fidler, 1933, 2]. From

this constancy we infer that the alcohol number may also be taken as a measure of N.R., and that the extinction point of N.R. will be that concentration of oxygen at which the alcohol number becomes zero.

Earlier workers by other methods have aimed at determining the extinction point of N.R. Thus Stich [1891], and several workers quoted by him, measured the effect of lowered oxygen concentration on the respiratory quotient of certain plant tissues, the general principle being that this quotient is increased when zymasis (*i.e.* N.R.) begins. None of these workers, however, measured alcohol.

Blackman [1928] inferred from CO_2 output curves that the extinction point of N.R. for Bramley's Seedling apples is below 5 % in early storage, and that it increases as the apples age. Again, no measurements of alcohol were made. The results we have obtained by using the alcohol number as an index are unambiguous, and they permit us to test whether the arguments based on data collected by other methods are valid. In order to compare the behaviour of the apples used by us with the behaviour of those used by Blackman and Parija [1928], we have also measured the rate of CO_2 production.

So far as we know, this is the first time that CO_2 and the alcohol number (or any other measure of accumulating zymasic products) have been simultaneously determined in gas mixtures of nitrogen and oxygen. Some of the advantages accruing from this procedure are developed later (see Section III). Thus, even when N.R. forms one component of the total CO_2 produced, what has been termed the total carbon loss [Blackman, 1928] has been calculated. Further, it is also possible by calculation to distribute total CO_2 between N.R. and normal respiration (Blackman's O.R.). The results of a few calculations are given in Section II in Table II, but we have not yet sufficient data for critical discussion. In fine, we consider that no analysis of glycolysis in which N.R. CO_2 is a product can approach completion unless cognisance is taken of the accumulating products of zymasis, should it be known that this process occurs under anaerobic conditions.

I. MATERIALS AND METHODS.

An account has already been given [Fidler, 1933, 1] of the times of gathering, and of the modes of storing and dispatching the fruit used in the experiments we are about to describe. The apparatus and methods of analysis employed have also been described by Fidler [1933, 1, 2].

Upon receiving the apples from storage the CO_2 output in air at 23° was determined. The air stream was then changed for one of pure nitrogen or of a nitrogen-oxygen mixture¹ at the rate of 3 litres per hour when the sample was a single apple, or 6 litres per hour when larger samples were used. Analyses of the issuing gas showed that the composition of the gas stream at this speed was not appreciably altered by the respiration of the fruit. Exposure to the artificial atmosphere lasted for about 4 days.

Sets of experiments were carried out at intervals during the storage season, the apples used in any one set being roughly of the same age. Each set consisted of two groups of four separate experiments, each of which was performed in a different concentration of oxygen. Six weeks to 2 months elapsed between each set of experiments. Thus, in addition to showing the effect of varying oxygen concentrations on apples of roughly the same age, it was also possible to deter-

¹ The nitrogen-oxygen mixtures were obtained in cylinders from the British Oxygen Company, Ltd., Walker Gate, Newcastle-on-Tyne, their composition being determined accurately by means of a Haldane gas analysis apparatus before use.

mine the effect of increasing age on zymasis by the fruit in any given concentration of oxygen. Work on a set of experiments lasted about 2 weeks, a new consignment of fruit for the second group of four experiments being timed to arrive on the day the apples used in the first four were distilled.

II. RESULTS.

A. *The alcohol number.*

Preliminary work [Fidler, 1933, 1] showed that the alcohol number of storage controls was not the best basis for evaluating change in the alcohol number owing to oxygen shortage; for it appears that zymasis may be induced by other factors connected with the experimental conditions. For gas mixtures containing oxygen, interest resides chiefly in the figures in column 11 of Table I. These have been obtained by deducting the corresponding conditions control values [Fidler, 1933, 1, Table II], from the alcohol numbers¹ found by analysis. There is no evidence that conditions as such increase zymasis in pure nitrogen, and for this gas the storage control has been used.

B. *The rate of CO₂ production, distributed between O.R. and N.R. Carbon loss.*

Only the results for the rate of production of CO₂ by Bramley's Seedling apples in 1931-2 will be considered here².

In Section III we compare our results for CO₂ output with those obtained by Parija [1928] who used Bramley's Seedling apples from a different locality. Our experiments differ from his in one important respect. To investigate the extinction point of N.R. he used the same apple in different concentrations of oxygen. Since we determined the alcohol number as well as the CO₂ output, we necessarily had to use different apples for every experiment.

NOTES TO TABLE I.

(a) *Weight of apple.* Up to experiment 62, the final weight was used in calculating the percentage concentration of products of zymasis. After this time the initial weight has been used. The loss in weight was never more than 6 % in a sound apple and was usually of the order of 1 to 3 %. As all the numbers in any series have been calculated to the same basis, the error is not serious.

(b) *Exps. 106, 107 and 108 on Bramley's Seedlings* were allowed to continue for about 8 days and then analysed. The alcohol numbers given in the table have been calculated by dividing the alcohol numbers obtained in the analysis by the quotient: CO₂ output per 8 days/CO₂ output during the first 100 hours.

(c) The results given for experiments in pure nitrogen are taken from Fidler [1933, 2], and are included for the sake of comparison with those obtained in 0.8 % oxygen.

(d) The storage control has been used in experiments where the alcohol number was greater than that in a conditions control.

¹ The ratio alcohol/acetaldehyde in these experiments was intermediate between 45/1 (the highest recorded in air) and 75/1 (the lowest recorded in nitrogen). It is not proposed to analyse these figures further since the research was not planned to determine the effect of oxygen tension and age of apples on this ratio. It is proposed, however, to carry out experiments to test these points in the near future.

² Results for Bramley's Seedlings in another season and for Newton Wonders in three seasons are given in a Ph.D. thesis [Fidler, 1932], where they have been treated in the manner adopted here for this selected set of results for Bramley's Seedlings.

Table I. *Changes in the percentage alcohol number for Newton Wonder and Bramley's Seedling apples in nitrogen-oxygen mixtures at 23°. (See notes to Table I.)*

No. of exp.	Variety of fruit	State of fruit	Date exp. begins	Hours' exposure to gas	Per-centage oxygen in gas	No. of apples in sample	Wt. of sample (g.)		After deduction of storage control value	After deduction of conditions control value	The percentage alcohol number after 100 hours
							Initial	Final			
Season 1930-31. From orchard											
15	Newton Wonder	Small. Green. Very young	22. vii. 30	123½	5.7	5	—	152.0	0.002	—	—
From cool storage											
17	Newton Wonder	Green. Hard	24. ix. 30	97½	Nil	1	—	112.5	0.19	—	—
18	Newton Wonder	Green. Hard	24. ix. 30	97½	3.6	1	—	93.5	—	Nil	—
19	Newton Wonder	Green. Hard	24. ix. 30	99½	5.7	1	—	113.5	—	Nil	—
20	Newton Wonder	Green. Hard. Young	1. x. 30	142	21.0	1	—	100.5	0.01	—	—
30	Newton Wonder	Green to green-yellow	27. xi. 30	97	Nil	1	—	193.0	0.25	—	—
31	Newton Wonder	Green-yellow	27. xi. 30	97	9.6	1	—	200.5	0.14	—	—
32	Newton Wonder	Green-yellow	27. xi. 30	97	3.6	1	—	200.0	0.08	—	—
37	Newton Wonder	Yellow. Slightly wilted	8. i. 31	99½	Nil	1	—	105.0	0.35	—	—
38	Newton Wonder	Green-yellow. Slightly wilted	8. i. 31	99½	2.6	1	—	142.5	0.28	—	—
39	Newton Wonder	Green-yellow. Slightly wilted	8. i. 31	99½	3.6	1	—	139.5	0.25	—	—
Season 1931-2. From orchard											
73	Newton Wonder	Green. Hard. Very young	1. vii. 31	97½	Nil	12	103.0	96.5	0.70	—	—
74	Newton Wonder	Green. Hard. Very young	1. vii. 31	97½	0.8	12	102.0	98.5	0.67	—	—
79	Newton Wonder	Green. Hard. Very young	17. vii. 31	113	21.0	6	134.5	132.5	0.01	—	—
80	Newton Wonder	Green. Hard. Very young	17. vii. 31	95	Nil	9	174.5	167.0	0.73	—	—
From cool storage											
81	Newton Wonder	Green. Hard. Very young	26. viii. 31	98½	Nil	3	208.0	201.0	0.38	—	—
82	Newton Wonder	Green. Hard. Very young	26. viii. 31	98½	3.0	3	213.0	212.5	—	Nil	—
83	Newton Wonder	Green. Hard. Very young	26. viii. 31	98½	5.0	3	203.0	198.0	—	Nil	—
84	Newton Wonder	Green. Hard. Very young	26. viii. 31	122	21.0	3	212.0	207.0	0.02	—	—
89	Newton Wonder	Green. Hard. Very young	10. ix. 31	99½	9.5	3	213.0	209.5	—	Nil	—
91	Newton Wonder	Green. Hard. Very young	10. ix. 31	99½	40.0	3	207.5	203.5	—	Nil	—
95	Newton Wonder	Green. Hard. Very young	10. ix. 31	99½	100	3	200.0	194.5	—	Nil	—
96	Newton Wonder	Green. Hard. Very young	28. x. 31	99	Nil	3	252.5	246.0	—	Nil	—
97	Newton Wonder	Green. Hard. Very young	28. x. 31	99	3.0	2	288.0	284.0	0.32	—	—
98	Newton Wonder	Green. Hard. Very young	28. x. 31	99	100	2	259.0	254.5	—	Nil	—
99	Newton Wonder	Green. Hard. Very young	28. x. 31	140	21.0	2	321.0	312.5	0.01	—	—
100	Newton Wonder	Green. Hard. Very young	4. xi. 31	98½	8.0	2	329.0	324.0	—	Nil	—
101	Newton Wonder	Green. Hard. Very young	4. xi. 31	98½	15.2	2	336.0	331.5	—	Nil	—
109	Newton Wonder	Green. Hard. Very young	4. xi. 31	98½	28.0	2	286.0	285.5	—	Nil	—
110	Newton Wonder	Green. Hard. Very young	10. xii. 31	98½	Nil	2	298.5	293.5	—	Nil	—
111	Newton Wonder	Green. Hard. Very young	10. xii. 31	98½	2.4	2	295.0	289.0	0.50	—	—
112	Newton Wonder	Green. Hard. Very young	10. xii. 31	98½	2.9	2	293.0	290.0	—	Nil	—
113	Newton Wonder	Green. Hard. Very young	10. xii. 31	121½	21.0	2	279.0	270.0	0.15	—	—
114	Newton Wonder	Green. Hard. Very young	16. xii. 31	99	8.5	2	299.0	295.5	—	Nil	—
115	Newton Wonder	Green. Hard. Very young	16. xii. 31	99	15.2	2	280.5	279.0	—	Nil	—
116	Newton Wonder	Green. Hard. Very young	16. xii. 31	99	100	2	281.0	278.5	—	Nil	—
125	Newton Wonder	Green. Hard. Very young	16. xii. 31	99	60	2	264.5	258.5	—	Nil	—
126	Newton Wonder	Green. Hard. Very young	27. i. 32	99	Nil	1	84.0	81.5	0.50	—	—
127	Newton Wonder	Green. Hard. Very young	27. i. 32	99	8.3	1	83.5	81.0	0.08	—	—
128	Newton Wonder	Green. Hard. Very young	27. i. 32	99	2.4	1	73.5	72.0	0.05	—	—
129	Newton Wonder	Green. Hard. Very young	27. i. 32	140½	21.0	1	97.5	97.0	0.02	—	—
130	Newton Wonder	Green. Hard. Very young	10. ii. 32	98	2.0	1	105.0	105.0	—	—	—
131	Newton Wonder	Green. Hard. Very young	10. ii. 32	98	5.3	1	92.5	91.5	0.17	—	—
133	Newton Wonder	Green. Hard. Very young	24. ii. 32	96½	60.0	1	86.5	84.5	0.02	—	—
	Newton Wonder	Green. Hard. Very young	24. ii. 32	138	21.0	1	87.0	83.5	0.02	—	—

Season 1930-31. From cool storage

25	Bramley's	Green.	Hard.	Young	19. xi. 30	97 $\frac{1}{2}$	Nil	—	148.5	0.34	—
26	Seedling	Green.	Hard.		19. xi. 30	97 $\frac{1}{2}$	2.6	—	147.5	0.08	0.05
27					19. xi. 30	99 $\frac{1}{2}$	5.3	—	149.0	0.03	0.001
34		Green.	Firm		10. xii. 30	97	Nil	—	124.5	0.31	—
35		"			10. xii. 30	99 $\frac{1}{2}$	2.6	—	153.5	0.17	0.16
43		Green to green-yellow			22. i. 31	98 $\frac{1}{2}$	Nil	—	134.5	0.37	—
44		Green-yellow.	Wilted		22. i. 31	98 $\frac{1}{2}$	2.6	—	150.0	0.25	0.17
45		Green-yellow.	Very slightly wilted		22. i. 31	98 $\frac{1}{2}$	5.0	—	118.5	0.18	0.10
46		Green-yellow.			29. i. 31	116	21.0	—	170.0	0.06	—
50		Yellow-green.	Slightly wilted		5. ii. 31	99 $\frac{1}{2}$	9.5	—	189.5	—	Nil
51		"			5. ii. 31	99 $\frac{1}{2}$	11.0	—	187.5	—	Nil
52		"			5. ii. 31	99 $\frac{1}{2}$	15.3	—	193.0	—	Nil
53		Yellow-green.			11. ii. 31	98	28.0	—	142.0	—	Nil
54		"			11. ii. 31	98	100	—	90.0	—	Nil
55		Yellow-green.	Badly wilted		5. iii. 31	116	21.0	—	147.0	0.15	—
57		Yellow.	Badly scalded and wilted		5. iii. 31	98	69.0	—	121.5	—	Nil
58		"			5. iii. 31	98	40.0	—	139.5	—	Nil

Season 1931-2. From orchard

71	Bramley's	Green.	Hard.	Very young	1. vii. 31	97 $\frac{1}{2}$	0.8	—	249.0	0.45	0.44
72	Seedling	Green.	Hard.	Very young	1. vii. 31	97 $\frac{1}{2}$	Nil	—	238.5	0.43	—
75		Green.	Hard.	Very young	8. vii. 31	116 $\frac{1}{2}$	21.0	—	172.0	0.01	—
77		Green.	Hard.	Very young	17. vii. 31	72 $\frac{1}{2}$	0.8	—	183.5	0.58	0.57
78		Green.	Hard.	Very young	17. vii. 31	95	Nil	—	179.0	0.60	—
85		"			2. ix. 31	99	Nil	—	200.0	0.40	—
86		Green.	Hard.	Very young	10. ix. 31	99	3.0	—	272.5	—	—
87		"			2. ix. 31	99	5.0	—	214.0	—	—
88		Green.	Hard.	Very young	2. ix. 31	99	21.0	—	236.0	0.01	—
92		Green.	Hard.	Very young	18. ix. 31	140 $\frac{1}{2}$	8.0	—	218.5	—	—
93		"			18. ix. 31	98	40.0	—	183.5	—	—
94		"			18. ix. 31	98	100	—	186.0	—	—

Season 1931-2. From cool storage

102	Bramley's	Green			11. xi. 31	98 $\frac{1}{2}$	Nil	—	223.0	0.50	—
103	Seedling	"			11. xi. 31	97	3.0	—	211.0	—	—
104	"	"			11. xi. 31	98 $\frac{1}{2}$	100	—	376.5	—	—
105	"	"			11. xi. 31	115 $\frac{1}{2}$	21.0	—	207.5	0.05	—
106	"	"			18. xi. 31	—	8.5	—	234.5	—	—
107	"	"			18. xi. 31	—	28.0	—	206.5	0.06	0.005
108	"	"			18. xi. 31	—	69.0	—	164.5	0.03	0.003
117	"	"			12. i. 32	99	Nil	—	187.5	0.32	—
118	"	"			6. i. 32	99	8.3	—	180.5	—	—
119	"	"			6. i. 32	99	2.4	—	112.0	—	—
120	"	"			6. i. 32	138 $\frac{1}{2}$	21.0	—	158.0	0.06	—
121	"	"			13. i. 32	99	0.8	—	181.0	0.34	0.28
122	"	"			13. i. 32	99	2.9	—	191.0	—	—
123	"	"			13. i. 32	99	5.2	—	148.0	—	—
124	"	"			13. i. 32	99	8.5	—	172.5	—	—
134	Green-yellow				6. iii. 32	97 $\frac{1}{2}$	Nil	—	138.0	0.50	—
135	"	"			16. iii. 32	97 $\frac{1}{2}$	8.3	—	134.5	—	—
136	"	"			16. iii. 32	97 $\frac{1}{2}$	2.4	—	247.5	—	—
137	"	"			16. iii. 32	140	21.0	—	167.5	0.10	0.02
138	Green-yellow.	First sign of wrinkling			31. iii. 32	97 $\frac{1}{2}$	2.9	—	164.0	0.09	—
139	"	"			31. iii. 32	97 $\frac{1}{2}$	5.3	—	97.5	0.11	0.07
140	"	"			31. iii. 32	97 $\frac{1}{2}$	60.0	—	138.0	0.05	0.001
141	"	"			31. iii. 32	116 $\frac{1}{2}$	21.0	—	152.0	—	—
								—	127.5	0.05	—

Table II. *Rate of CO₂-production, N.R., O.R., carbon loss, and CO₂/alcohol number of Bramley's Seedling apples during 1931-2. (For details of state of fruit, date of experiment and the alcohol number, see Table I.)*

g./100 g. apple/100 hours								
Exp. No.	Oxygen in gas %	Actual carbon loss*	Actual CO ₂ output	Corrected for conditions control values†				Total carbon loss Carbon lost as CO ₂
				CO ₂	N.R.	O.R.	CO ₂ /alcohol number	
1	2	3	4	5	6	7	8	9
From orchard:								
71	0.8	0.42	0.680	0.67	0.50	0.17	100:63	
72	Nil	0.42	0.714	—	0.71	Nil	100:58	2.3
75	21.0	0.15	0.542	0.54	—	0.54	—	2.2
77	0.8	0.50	0.727	0.72	0.64	0.07	100:76	1.0
78	Nil	0.50	0.667	—	0.67	Nil	100:93	2.6
85	Nil	0.33	0.455	—	0.46	Nil	100:83	2.7
86	3.0	0.05	0.176	0.16	Nil	0.16	—	2.6
87	5.0	0.07	0.239	0.22	Nil	0.22	—	1.0
88	21.0	0.13	0.446	0.43	—	0.43	—	1.0
92	8.0	0.06	0.200	0.19	Nil	0.19	—	1.0
93	40.0	0.06	0.203	0.19	Nil	0.19	—	1.0
94	100	0.10	0.337	0.32	Nil	0.32	—	1.0
From cool storage:								
102	Nil	0.46	0.592	—	0.59	Nil	100:80	
103	3.0	0.14	0.390	0.33	Nil	0.33	—	2.6
104	100	0.18	0.563	0.50	Nil	0.50	—	1.0
105	21.0	0.18	0.545	0.49	—	0.49	—	1.0
106	8.5	0.16	0.477	0.42	Nil	0.42	—	1.0
107	28.0	0.17	0.469	0.41	0.005	0.41	—	1.0
108	69.0	0.20	0.605	0.55	0.004	0.54	100:3	1.0
117	Nil	0.30	0.456	—	0.46	Nil	100:1	1.0
118	8.3	0.15	0.419	0.35	Nil	0.35	100:67	2.4
119	2.4	0.15	0.414	0.35	Nil	0.35	—	1.0
120	21.0	0.20	0.594	0.54	—	0.54	—	1.0
121	0.8	0.33	0.520	0.46	0.46	Nil	—	1.0
122	2.9	0.14	0.384	0.32	Nil	0.32	100:58	2.2
123	5.2	0.17	0.465	0.40	Nil	0.40	—	1.0
124	8.5	0.19	0.539	0.47	Nil	0.47	—	1.0
134	Nil	0.45	0.631	—	0.63	Nil	—	1.0
135	8.3	0.18	0.496	0.44	Nil	0.44	100:76	2.6
136	2.4	0.20	0.490	0.42	0.02	0.40	—	1.0
137	21.0	0.21	0.567	0.57	—	0.52	100:4	1.0
138	2.9	0.22	0.499	0.44	0.09	0.36	—	1.0
139	5.3	0.18	0.493	0.44	0.006	0.43	100:14	1.6
140	60.0	0.22	0.615	0.57	Nil	0.57	100:1	1.0
141	21.0	0.22	0.638	0.59	—	0.59	—	1.0

* *I.e.* total gaseous loss.

* *I.e.* total zymasis in carbon units (carbon equivalent of column 4, plus the carbon equivalent of the conditions control alcohol number).

† Except in Exps. 72, 78, 85, 102, 117, 134, where O₂ % = Nil.

NOTES TO TABLE II.

(a) *Distribution of CO₂ between O.R. and N.R.* Before distributing the CO₂ given off in gas mixtures of nitrogen and oxygen between O.R. and N.R., allowance must be made for the CO₂ component of zymasis which is induced by conditions as such (see p. 1631). This part of the total CO₂ produced may be calculated from the alcohol number of the conditions control.

For this calculation, instead of using the conventional equation for alcoholic fermentation, we prefer to use the formula experimentally determined for the variety of apple under observation. Strictly, this formula should be determined each season, but there are insufficient data for this refinement. Instead we take the mean value for the given variety for all the seasons during which

this apple has been used. Thus the preliminary work over two seasons [Fidler, 1933, 2] permits us to write for Bramley's Seedlings: respirable substrate $\rightarrow 100 \text{ CO}_2 + 85 \text{ C}_2\text{H}_6\text{O}$. By subtracting this CO_2 component of zymesis occurring in air at 23° under experimental conditions from the total CO_2 absorbed by the baryta, we arrive at a number representing the CO_2 which must be distributed between O.R. and N.R.

If the alcohol number obtained in any experiment is of the same order as that of a conditions control, we conclude that there is no N.R. due to oxygen shortage. All the CO_2 produced is then attributed to O.R.

When, however, zymesis due to oxygen shortage is detected, the CO_2 component is calculated as we have described above. Thus, for Bramley's Seedlings, in calculating N.R. due to oxygen shortage, 100 g.-mol. equivalents of CO_2 are taken as equivalent to 85 g.-mol. equivalents of zymasic products accumulating in the tissues. The O.R. occurring simultaneously may be found by subtracting the calculated value for N.R. from the total CO_2 output corrected for experimental conditions.

(b) *Calculation of the percentage carbon loss.* The percentage carbon loss, given in Table II, is the sum of the weights in g. of carbon given off as CO_2 and excreted in the tissues as ethyl alcohol and acetaldehyde.

(c) *The quotient Total carbon loss/Carbon lost as CO_2 .*

In the paper dealing with zymesis in nitrogen [Fidler, 1933, 2], a column was included giving Glycolysis in C units/N.R. in C units. Such a column cannot be included in the present paper, since total carbon loss in nitrogen-oxygen mixtures is not equal to glycolysis in carbon units. The difference is due to O.A. (oxidative anabolism [Blackman, 1928]). This, however, cannot be evaluated from our data. A column has been added here giving the quotient Total carbon loss/Carbon lost as CO_2 . When there is no N.R. the value of this quotient is unity. As N.R. forms an increasingly important component of respiration, the quotient rises to a maximum of 2.7 (for an anaerobic CO_2 /alcohol ratio of 100/85).

III. DISCUSSION AND GENERAL CONCLUSIONS.

(A) *The effect of varying oxygen concentrations on the alcohol number in Newton Wonder and Bramley's Seedling apples of different ages and in different seasons.*

(i) *The alcohol number as a measure of N.R., and the possible relations between N.R. and oxidation processes.* The graphs in Figs. 1a and 1b, and 2a and 2b¹, in which are represented the results given in Table I for Newton Wonder and Bramley's Seedling apples, show the concentrations of oxygen that extinguish N.R. at different times in the storage season, and what is the relative extent of N.R. when it occurs. When the alcohol number points lie above the x-axis, zymesis takes place to a greater extent than in a conditions control [see Fidler, 1933, 1]. It follows first that N.R. then forms one component of the respiratory process, and, secondly, that this N.R. occurs owing to a deficiency of oxygen in the gas mixture passed over the fruit. Further, the relative magnitudes of N.R. in the total respiration in different gas mixtures² may, of course, be

¹ To avoid crowding of graphs we have assembled in Figs. 1a and 1b the results for apples before they became pronouncedly yellow, and in Figs. 2a and 2b the results for the same populations when they were rapidly becoming or had become yellow. This colour change criterion of the ageing of the flesh tissue of apples has been used before [see e.g. Blackman and Parija, 1928]. Evidence given later in this section suggests, however, that other phenomena of ageing do not necessarily march in line with this colour change, i.e. apples of the same variety, from the same population and of the same colour are not necessarily in the same physiological state in all respects.

² In column 9, Table II in Section II, it will be seen that the ratio Total carbon loss/Carbon lost as CO_2 decreases from 2.7 to unity when the oxygen concentration is increased. The extinction point is the minimum oxygen concentration at which this ratio becomes unity.

judged from the differences in the heights of the points above the x -axis. When, however, the alcohol number points lie on the x -axis, there is no zymasis beyond that occurring in a conditions control. In these cases there is sufficient oxygen in the gas mixtures to extinguish N.R. completely.

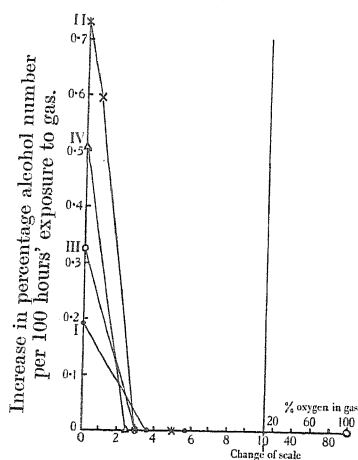


Fig. 1a.

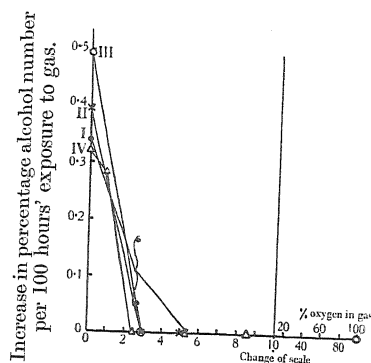


Fig. 1b.

Fig. 1a. Percentage alcohol numbers of green to yellow-green Newton Wonder apples.

Season 1930-1. From store: I, 24. ix. 30. ●
 Season 1931-2. From orchard: II, 17. vii. 31-26. viii. 31. ×
 From store: III, 28. x. 31. ○
 From store: IV, 10. xii. 31. △

Fig. 1b. Percentage alcohol numbers of stored green to yellow-green Bramley's Seedling apples.

Season 1930-1. I, 19. xi. 30-10. xii. 30. ●
 Season 1931-2. II, 2-10. ix. 31. ×
 III, 11. xi. 31. ○
 IV, 6-13. i. 32. △

The graphs afford unquestionable evidence that at all times in the storage season, as the oxygen concentration outside the apples is increased, N.R. gradually falls from the maximum value attained in pure nitrogen. In most of our experiments this fall in N.R. continued until zymasis completely ceased: then, of course, the extinction point of N.R. is the minimum oxygen percentage at which the alcohol number reaches zero. This percentage is not the same for all apples (see later sub-sections).

We have insufficient data for low concentrations of oxygen to calculate the changing quantitative relations between N.R. and oxidation processes as the oxygen concentration rises (see also footnote on p. 1640). The most obvious hypothesis is that N.R. is substituted step by step by oxidation processes in rising oxygen concentrations. There are two conflicting contemporary views that would then have to be borne in mind in explaining the inhibitory action of oxygen on zymasis. According to one view, N.R. and oxidative processes are cognate. Accepting this view, we might infer from the experiments and arguments of Meyerhof [1925] and Blackman [1928] that, as nitrogen is progressively replaced by oxygen, the products of glycolysis are diverted from ethyl alcohol and CO_2 (N.R.) to water and CO_2 (O.R.) and O.A. (oxidative anabolism,

or the Meyerhof-Pasteur reaction). According, however, to the alternative view (see for example Lundsgaard [1930] and Boysen-Jensen [1922; 1931], the enzyme systems governing N.R. and oxidation processes are quite distinct. Should this view become established it would mean that the contiguous processes of N.R. and oxidation occurring in low concentrations of oxygen are unconnected although they affect the same substrate. It would then follow

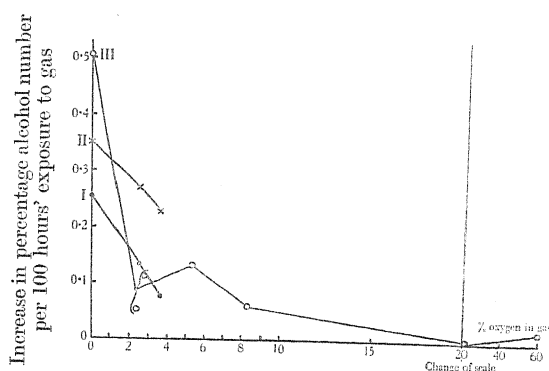


Fig. 2a. Percentage alcohol numbers of green-yellow to yellow Newton Wonder apples.

Season 1930-1. I, 27. xi. 30. ●
 II, 8. i. 31. x
 Season 1931-2. III, 27. i. 32-24. ii. 32. ○

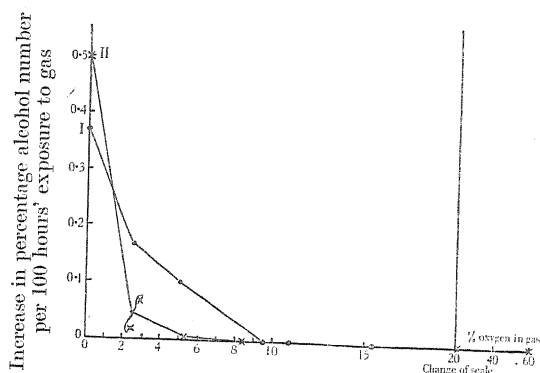


Fig. 2b. Percentage alcohol numbers of green-yellow to yellow Bramley's Seedling apples.

Season 1930-1. I, 22. i. 31-5. ii. 31. ●
 Season 1931-2. II, 16-31. iii. 32. x

that in rising oxygen concentrations zymasis is increasingly inhibited and that simultaneously oxidation processes, which are independent of anaerobic glycolysis, are activated to an equivalent extent.

(ii) *The extinction point of N.R. early in the storage season.* In Fig. 1a are recorded the alcohol numbers for Newton Wonder apples during two seasons at the beginning of the cool storage periods. When these experiments were started we thought that zymasis would occur in all concentrations of oxygen

less than 5 %. But our results for this variety indicate that early in storage zymasis, owing to oxygen shortage, does not occur even in 2.5 % oxygen¹. For Bramley's Seedlings, in 1931-2, it will be seen from graphs II, III and IV in Fig. 1*b*, that this same concentration of oxygen completely inhibited zymasis in September and November 1931, and even as late as January 1932. At this last date the apples which we used were yellow-green². Our results for this same variety at the corresponding time in the previous season cannot be used in the present analysis; for before the middle of December 1930 (see graph I, Fig. 1*b*) the apples, whilst still green, underwent zymasis in all gas mixtures containing less than 5 % of oxygen. It is probable, however, that had we experimented on Bramley's Seedlings at an earlier date in the autumn of 1930, zymasis would have been stopped by a lower concentration of oxygen (see next subsection).

We conclude, therefore, that the extinction point of N.R. for apples of these two varieties whilst they are still on the tree or early in storage is not greater than 2.5 % oxygen. This is a surprisingly low figure. And it must be remembered that in a bulky organ, such as an apple, the internal concentration of oxygen in the intercellular spaces around the respiring cells is always less than the concentration of oxygen outside. Questions concerning the oxygen relations of possible respiratory systems in cells suggest themselves, but must for the present be left unanswered.

(iii) *The shift of the extinction point in ageing apples.* The graphs in Figs. 2*a* and 2*b* show that zymasis is not completely inhibited by 2.5 % oxygen when senescent characteristics, such as the change to green-yellow and yellow, wilting and superficial scald, appear. Thus we conclude that although the extinction point of N.R. appears to be constant early in the storage season, it shifts to higher values when signs of ageing become more marked.

The charts for the preliminary experiments on green-yellow Newton Wonder apples in 1930-1 (Fig. 2*a*, graphs I and II) show that, notwithstanding the steep decline in the alcohol numbers with increasing oxygen concentrations, zymasic products accumulated in apples in 3 % oxygen in greater amounts than in the conditions controls. Early in 1932 experiments were carried out with a wider range of gas mixtures and we interpret graph III as showing that after January, in green-yellow air-stored Newton Wonder apples, (a) N.R. is not completely extinguished in any concentration of oxygen (*cf.* the complete extinction of N.R. in 3 % oxygen in December 1931—graph IV, Fig. 1*a*) and (b) for each increase in oxygen concentration up to a value that is less than 5 % oxygen, the corresponding decrease in N.R. is more rapid than when the oxygen concentration is increased beyond 5 %. We are not prepared, from this experiment alone, to state what is the precise form of the curve representing this fall in N.R. in senescent apples.

¹ Graph II in Fig. 1*a* shows that very young green apples picked from orchard trees behave similarly to fully grown green apples taken from store.

² Although colour is a useful guide for grouping apples, it is clearly not a rigid criterion of physiological state. Here we see that zymasis occurred in green apples in December 1930 in 2.5 % oxygen but was completely inhibited by this concentration in yellow-green apples in the next season. On a colour standard these latter apples would be considered to have been the more senescent of these two samples, but in terms of the respiratory events in the cells of the flesh tissue we consider that they were the less senescent. There is an element of hazard at present in arranging comparable experiments for stored apples in two different seasons. This might conceivably be lessened by using in successive seasons only those apples that have approximately the same extinction point of N.R.

In the comparable experiments with Bramley's Seedling apples (Fig. 2*b*), we note from graph I that in late January to early February 1931 as much as 9 % oxygen was needed to extinguish N.R. But when we examine graph I in Fig. 1*b*, we see that in December 1930, *i.e.* about 6 weeks earlier in the storage season, zymasis was nearly completely checked by only 5 % oxygen. For Bramley's Seedling apples ageing in air-stores during 1930-1, as well as for Newton Wonder apples in this same season, there is clear evidence, therefore, that in late storage greater concentrations of oxygen are needed to inhibit zymasis, *i.e.* to extinguish N.R., than in early storage.

In 1931-2, the results for Bramley's Seedlings, although not so striking, point to the same conclusion. Thus graphs II, III and IV in Fig. 1*b* show quite definitely that there was no zymasis in concentrations greater than 3 % oxygen before January 1932; and from graph II in Fig. 2*b* we see that by March 1932 the alcohol number had risen distinctly above the conditions control value in 3 % oxygen. At this latter date the slight rise shown in 5.3 % oxygen is by itself hardly significant, but as this rise is quite in accord with the behaviour of Bramley's Seedlings in the previous season, and with Newton Wonders in both seasons, it strengthens the evidence for a shift in the extinction point of N.R. to higher levels in the later phases of storage.

Blackman analysed a series of curves of CO₂ output by Bramley's Seedlings in gas mixtures containing different percentages of oxygen [Blackman, 1928] and inferred that such a shift occurs. Using the alcohol number as an index of N.R. we have verified Blackman's inference by our work on two varieties of apple.

(iv) *Varietal and seasonal differences as to the date at which the shifts of the extinction point begins.* We may first note that different varieties from the same orchard and picked at about the same time, and with a similar storage history, may behave differently in a given storage season. For example, graph II in Fig. 2*b* records but little zymasis in March 1932 in Bramley's Seedlings at concentrations of oxygen above 3 %. We conclude that the extinction point had just started to shift at this date. In striking contrast, the shift for Newton Wonders was well under way a month earlier. Indeed, for this variety, at this time, N.R. was not completely extinguished in quite high concentrations of oxygen. This may be because the senescent breakdown of the respiratory centres in the protoplasm of the cells had begun [see Fidler, 1933, 1]. This is possibly the inevitable fate of all apples kept in store, and it is conceivable that the first shift of the extinction point may be a sign of incipient disorganisation. Should this turn out to be the case, varietal differences in regard to this shift may prove to be related to the different keeping qualities of different varieties in a given season.

Secondly, variation has been observed in the behaviour towards oxygen of a given variety of apple picked from the same orchard in successive years at approximately the same time and stored and dealt with each year as nearly as was practicable in the same fashion. Fig. 2*b* clearly illustrates this seasonal difference for Bramley's Seedlings. A comparison of graphs I and II shows that the extinction point had shifted far more by February 1931 than by March in the following storage season. In fact this shift by March in the second season was no greater than that which had occurred by December in the previous season (see graph I, Fig. 1*b*). We infer that the date on which the seasonal shift in the extinction point begins is, for any given variety, determined by the impress made by the complex of significant environmental factors incident on the fruit whilst it is still on the tree. Kidd and West [1919-31] in their storage

trials have frequently reported that the impress of these factors persists in storage apples and governs their keeping qualities. There may be a relation between these qualities and the time at which the shift of the extinction point begins; and we found that Bramley's Seedlings kept better in 1931-2 than in 1930-1. The more rapid deterioration of this variety in the first season was possibly owing to the earlier shifting of the extinction point.

B. The rates of CO₂ output and of carbon loss in relation to oxygen concentration.

(i) *CO₂ output.* Blackman [1928] stated that the extinction point of N.R. is reached in that concentration of oxygen where CO₂ production is at a minimum, and he placed this minimum at 5 % oxygen. Our results accord well with his. Thus the graphs in Fig. 3 show that at all dates in 1931-2, as the oxygen concentration in the external atmosphere was increased, the rate of CO₂ output by Bramley's Seedlings first fell from the high value attained in nitrogen to a minimum value and then rose to the normal rate for air¹. The extinction point again (see sub-section A) appears to be less than 5 % oxygen.

A few points are given for higher concentrations of oxygen. These suggest the continued upward drift reported by Blackman [1930].

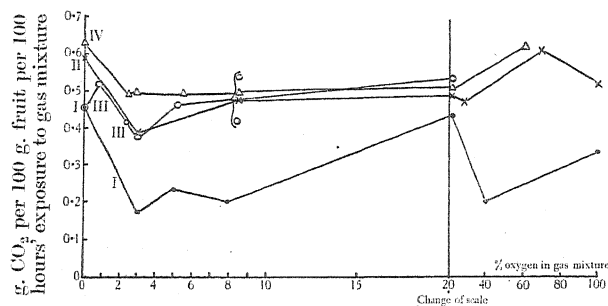


Fig. 3. CO₂ output by Bramley's Seedling apples during 1931-2. Corrected for conditions control values.

I, 2-18. ix. 31. ● III, 6-13. i. 32. ○
II, 11-18. xi. 31. × IV, 16-31. iii. 32. △

Blackman concluded from the seasonal variation in the oxygen concentration at which the minimum CO₂ output was observed, that the extinction point for N.R. would move to higher oxygen concentrations as the fruit aged. Our conclusions in sub-section A support Blackman's views. Further, we note here that we have found for Newton Wonders in 1930-1 and 1931-2, and for Bramley's Seedlings in 1930-1, that the oxygen concentration in which the minimum value for CO₂ output was observed rose from 3-5 % oxygen to 8-10 % oxygen as the apples aged². This rise affords further evidence that the behaviour of the apples we have used was substantially the same as that of the Bramley's Seedlings used by Blackman and Parija.

¹ The distribution of the CO₂ produced between N.R. and O.R. has been calculated, and the resulting values are given in Table II; but we have not collected enough data yet for gas mixtures containing less than 2.5 % oxygen to permit of critical analysis. We propose to perform further experiments in these low percentages of oxygen.

² The data on which this statement is based are included in Fidler's Ph.D. thesis [1932].

(ii) *Carbon loss.* In view of Blackman's calculations [1928] further data on the rate of carbon loss in relation to oxygen concentration may prove of interest.

We have obtained values for the rate of carbon loss under different conditions by simultaneously measuring CO_2 output and the alcohol number. We note that each carbon loss graph in Fig. 4 has the same form as the corresponding CO_2 output graph and has the same position for the minimum, *viz.* at about 3–5 % oxygen, the extinction point of N.R.¹ Carbon loss is greatest under anaerobic conditions. As the oxygen concentration passes from zero upwards, we observe that the first effect of oxygen is increasingly to restrict carbon loss. This restriction can be correlated with the gradual inhibition of zymasis as the oxygen concentration rises. Carbon loss appears to be at a minimum when zymasis has been completely inhibited. The second effect of oxygen, *viz.* to increase carbon loss by stimulating respiration, shows clearly after this minimum (see Blackman [1928]).

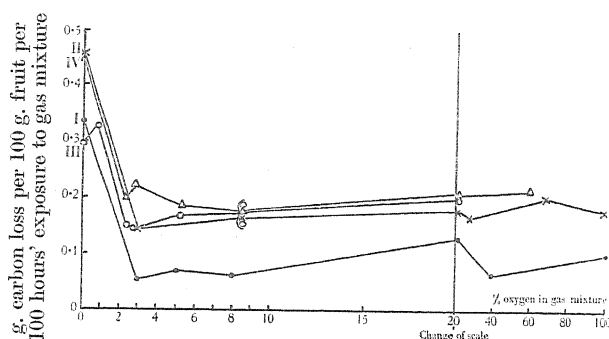


Fig. 4. Actual carbon loss by Bramley's Seedling apples during 1931–2.

I, 2–18. ix. 31. ●
II, 11–18. xi. 31. ×

III, 6–13. i. 32. ○
IV, 6–13. iii. 32. △

It will be noticed that the graphs I, II, III and IV lie at higher levels as the season advances. In all concentrations of oxygen a general seasonal stimulation of respiration probably contributes to this observed rise in carbon loss. In the lower concentrations of oxygen, moreover, the restricting effect of oxygen on zymasis may diminish.

SUMMARY.

Apples have been exposed to mixtures of nitrogen and oxygen at 23° at different times during the developmental and storage life of the fruit. Using conditions controls rather than storage controls, and, allowing for experimental error and for sampling variation, we have found that:

(i) Rising oxygen concentrations progressively retard zymasis. The high rate attained in pure nitrogen falls until at a certain concentration of oxygen no alcohol is produced. This concentration is termed the extinction point of N.R. (nitrogen respiration).

(ii) Early in the storage season in Newton Wonder and Bramley's Seedling apples the extinction point lies between 1 and 3 % oxygen. There is some evidence that it may be near to 3 % oxygen.

¹ It was shown by Fidler [1932] that the same agreement holds between the extinction point of N.R. and the minimum carbon loss, early and late in the season, for (a) Bramley's Seedlings 1930–1, (b) Newton Wonders during three seasons.

(iii) The extinction point shifts to higher concentrations of oxygen later in the storage season.

(iv) In old apples alcohol may accumulate even in 100 % oxygen, *i.e.* there is no extinction point for N.R.

(v) The time of the beginning of the shift of the extinction point may be different for different varieties in the same season and for a single variety in different seasons.

(vi) The rates of CO₂ production and of carbon loss fall from maximum values in nitrogen to minimum values in 3-5 % oxygen early in the storage season, but in 8-10 % oxygen for older apples. The rates increase gradually when the oxygen concentration is progressively raised above these critical concentrations.

This research was performed under the auspices of the Department of Scientific and Industrial Research [see Fidler, 1933, 1]. Grants by the Royal Society and by the Research Committee, Armstrong College to one of us (M. T.) have helped on the research.

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CCXXIII. THE DIFFERENTIAL INACTIVATION OF INSULIN.

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(Received July 19th, 1933.)

ALTHOUGH one of the most characteristic effects of insulin administered to the normal animal is a reduction of the blood-sugar content it has also been amply demonstrated that the concentrations of inorganic phosphate [Wigglesworth *et al.*, 1922-23] and amino-acid-nitrogen [Luck *et al.*, 1928] in the blood are also reduced. These demonstrations have been made not only with the ordinary preparations of insulin used therapeutically but also with crystalline insulin.

The object of the experiments now to be discussed was to determine whether or not these three characteristics of insulin activity are functions of the entire molecule acting as a unit or of distinct portions of the molecule, acting more or less independently. In support of the unitary hypothesis is the common belief that phosphate-lowering, following insulin, is secondary to the hypoglycaemia, or at least intimately associated with the well-recognised changes in carbohydrate metabolism. Nevertheless, no satisfactory proof of such a relationship has ever been advanced. Nor is there any evidence that the effect of insulin upon the amino-nitrogen content of the blood is dependent upon the carbohydrate changes. It occurred to us that the possible existence of an inter-relationship between these three physiological activities might be investigated directly through the use of partially inactivated preparations of the hormone.

It would be superfluous to discuss here in detail the extensive investigations of Freudenberg and associates, of Jensen, Scott, Du Vigneaud and others on insulin inactivation which have contributed much to our knowledge of the so-called "active groups" of insulin. In general, these investigations have been restricted to the chemistry of inactivation, employing as the sole criterion of activity the ability of the material to lower the blood-sugar content or, in large doses, to induce convulsions.

The present investigation is essentially an extension of such studies, the treated insulin being examined, not only in respect of its effect upon blood-sugar content but upon inorganic phosphate and amino-acid-nitrogen as well.

EXPERIMENTAL.

Inactivation of insulin. Two methods of inactivation were employed. In the first, crystalline insulin¹ was treated at 0° with 0.75N HCl in 75 % alcohol according to the method of Carr *et al.*, [1929]. 0.5 mg. samples were weighed

¹ We are much indebted to Dr D. A. Scott of the Insulin Laboratories, Toronto, and Prof. Du Vigneaud of George Washington University for the crystalline insulin used in this investigation.

out on a micro-balance and dissolved in 1 cc. portions of the acid-alcohol. The solutions so obtained were diluted to 12 cc. after 0, 1, 2, 3, 4, 5, 6, 7 or 8 days and thereupon injected at once into fasting rabbits.

In the second method, soft X-rays of high intensity were used as the inactivating agent. 0.2 to 0.5 cc. portions of insulin, 80 units per cc., were measured out into cylindrically ground depression slides, having a capacity of about 0.5 cc. The slides were sealed with thin mica cover slips (of known thickness) and vaseline and irradiated for periods ranging from 1 second to 2 hours.

The apparatus employed has been used by one of us (J. M. L.) in other experiments [Brown *et al.*, 1933] and was designed to give radiation of unusual intensity through a short target to specimen distance (3 cm.), silver anode, thin aluminium window (0.003 inch) and moderately high power (30 ma. at 50 kv.). The intensity as measured by an ionisation chamber approximated to 2500 Roentgen units per second¹.

Immediately after irradiation the specimens were diluted to 16–40 cc. (1 unit per cc. based on initial activity) and injected into fasting rabbits².

Experimental animals. Large female rabbits of 3–3.5 kg. weight, previously maintained upon a diet of barley and alfalfa were used. The animals were deprived of food for 2 days prior to injection.

Blood analysis. Blood samples, each of 5 cc. were collected, the first shortly before injection, the others at suitable intervals thereafter. In most of the experiments on inactivation by acid-alcohol only a single post-injection sample was drawn in order to minimise the chances of haemorrhage itself exerting an effect and thus leading to possible misinterpretation of results. In the rest of the acid-alcohol experiments, samples were drawn at 0, 1, 2, 3, 4, 5 and 6 hours. In all the X-ray experiments, the times of collection were 0, 1.5 and 3 hours.

Samples were analysed for reducing sugar, inorganic phosphate and amino-acid-nitrogen by the methods of Folin [1928], Fiske and Subbarow [1925] and Folin [1922] respectively.

RESULTS.

In Table I we have presented the results of experiments on 85 rabbits, injected with preparations of crystalline insulin which had been treated at 0° with 0.75N HCl in 75 % ethyl alcohol. Since it is not feasible to present the individual results, we have averaged the values within groups and reduced the averages so obtained to a common base line. This facilitates comparison.

Percentage retention of activity has also been calculated according to Freudenberg's method [Freudenberg and Dirscherl, 1928] and the values incorporated in Table I. Reference to this portion of Table I permits the conclusion that after 2 or 3 days of treatment at 0° with acid-alcohol, 25–30 % of the amino-nitrogen-lowering activity is retained, 15–50 % of the sugar-lowering activity and 60–80 % of the phosphate-lowering activity. The ability of insulin to lower the inorganic phosphate content of blood seems to be most resistant to inactivation by this means.

To obtain more frequent samples 27 additional experiments were performed, in which blood-samples were collected at 0, 1, 2, 3, 4.5 and 6 hours after

¹ We wish to acknowledge, most gratefully, the aid of Mr Morden G. Brown in operating the X-ray equipment.

² Prompt use of the irradiated insulin is important. Long-irradiated specimens, permitted to stand overnight, were found to be completely inactive with respect to amino-acid-nitrogen, sugar and phosphate. It is possible that hydrogen peroxide formed on irradiation causes this secondary destruction.

Table I. *Inactivation of insulin with acid-alcohol at 0°.*

Post-injection changes in blood composition.

Time of inactivation (days)	Number of rabbits	Three-hour post-injection values.			Percentage of initial activity retained*		
		Percentage of initial			Amino-N activity	Sugar-lowering activity	Phosphate activity
		Amino-N	Sugar	Phosphate			
0	12	80	58	76	100	100	100
1	11	89	73	74	54	64	100
2	8	95	81	80	25	52	84
3	13	93	94	83	32	14	63
5	12	—	94	92	—	16	32
7	17	100	92	92	0	22	34
8	6	98	101	85	14	0	53
9	6	93	103	98	32	0	11
Control†	11	101	99	98	—	—	—

* Activity of untreated insulin regarded as 100. The following formula was used:

$$\text{Percentage of initial activity retained} = \frac{100(a-b)}{c}$$

where a = normal concentration of amino-N, phosphate, or reducing sugar in blood; b = average concentration of given constituent 3 hours after injection with treated insulin; c = average reduction in concentration of the given constituent 3 hours after injection with untreated insulin.

† Insulin not injected.

injection. The periods of treatment with acid-alcohol were 1, 3, 5 and 8 days. The results, expressed as concentrations of the various blood constituents after 0, 1, 2, 3, 4.5 and 6 hours, were averaged by groups and plotted.

In computing the percentage retentions of activity the area over each curve was calculated and the values obtained compared with the reduction given by untreated insulin. Corrections were made, necessarily, for the slight hypoaminoacidaemia and hypophosphataemia produced by bleeding (control experiments without insulin).

The results were qualitatively the same as those presented in Table I. After 5 days of treatment with acid-alcohol only 12 % of the sugar-lowering activity was retained, in contrast with 82 % of the phosphate activity.

In Table II the results of the experiments on inactivation with soft X-rays are collected. After 15 minutes of irradiation only about 25 % of the amino-acid lowering activity remained but this fraction persisted undiminished even

Table II. *Inactivation of insulin with soft X-rays.*

Time of inactivation (mins.)	Number of rabbits	Post-injection values after 1.5 and 3 hours.						Percentage of initial activity retained		
		Percentage of initial						Amino- acid activity	Sugar activity	Phos- phate activity
		Amino-acids		Sugar		Phosphate				
		1.5	3	1.5	3	1.5	3			
0	10	87	77	54	55	78	79	100	100	100
0.004-0.25	10	94	89	55	55	77	70	43	99	110
0.5-4.0	6	88	87	58	60	74	69	75	91	118
15	8	93	93	79	91	86	87	29	37	60
30	6	98	94	81	95	88	87	20	31	55
60	6	96	94	104	107	92	88	29	- 11	40
120	4	97	95	102	100	94	85	23	- 2.4	41
120*	2	96	86	106	120	91	79	45	- 24	56
120†	2	104	97	111	121	93	86	9	- 30	41

* 5 × dose.

† 10 × dose.

after 2 hours' exposure. After 30–60 minutes of irradiation the hypoglycaemic activity of the insulin was lost completely. In fact, long-irradiated specimens tended to be hyperglycaemic—a property which became more evident when the irradiated insulin was injected in massive doses. As for phosphate-lowering activity, again we observed that this property of the hormone was the most resistant towards destruction. Even after 2 hours of irradiation, from 40 to 50 % of the initial activity remained. This lowering of phosphate, obtained in the absence of hypoglycaemia, demonstrates that a substantial portion of the activity of insulin towards phosphate cannot be secondary to the carbohydrate activity of the hormone.

Table II also contains the results of several experiments in which massive doses of the long-irradiated insulin (2 hours) were injected. The object here was to determine whether these larger doses might not increase, substantially, the changes in concentration in the blood of the substances studied.

Although the amino-acid changes were inconclusive and the decrease in phosphate activity remained at 50 or 60 %, the most interesting observation was the unmistakable tendency towards hyperglycaemia. Doubtless several explanations can be advanced to account for this phenomenon. However, we are disposed to think that the irradiated insulin induces an increased secretion of adrenaline. If so, the persistent though lessened activity towards amino-acids and phosphate may be due to adrenaline.

As an incidental observation, it should be mentioned that these massive doses of irradiated insulin failed to induce convulsions.

DISCUSSION.

All the experiments described in this paper agree in showing that the phosphate-lowering activity of insulin is the most resistant to destruction by the methods of inactivation examined. They demonstrate that insulin may be differentially inactivated—in the sense that the physiological activities of insulin may be separately destroyed. A complete separation was not accomplished, inasmuch as the loss of hypoglycaemic activity was accompanied by a loss of 50 % or so of the phosphate-lowering activity. Nevertheless it is evident that these three activities of the hormone are not lost in unison as the unitary hypothesis¹ of insulin structure and function would demand. The results permit no final conclusion as to the relative ease of destruction of the amino-acid-lowering activity, although our experiments with brief periods of X-ray irradiation frequently indicated that this factor is less stable to X-rays than the carbohydrate-lowering factor.

Here it should be mentioned that controls have been run with untreated insulin administered in dosages of 1 to 1/16 unit per kg. in order to see the effect of mere insulin dilution upon the blood-constituents¹. We had in mind the possibility that the "differential" inactivation here reported might be more apparent than real; that inactivating agents, instead of affecting one kind of active group to a greater extent than another, might inactivate completely a fraction of all the molecules present—leaving the others unaltered. Such an explanation would be demanded by the unitary hypothesis. We found, however, that reduction of the quantities of insulin administered caused the activities with respect to blood-sugar and inorganic phosphate to be "diluted-out" in

¹ By the "unitary hypothesis" we mean that theory of insulin structure and function which postulates that the various physiological activities of insulin are the property of a single active "centre" or "unit."

² The experiments with these lower dosages of insulin were made by Mr Walton Van Winkle.

unison. These results will be included in a later paper of this series and need not be discussed further at this time. There was no indication, however, that low doses of insulin (comparable with a mixture of active and inactive molecules resulting from inactivation) would lower blood-phosphate without causing hypoglycaemia.

With respect to the X-ray experiments it is pertinent to mention that earlier attempts at inactivation (sugar-lowering activity) with X-rays have given only negative results. Den Hoed *et al.*, [1929] irradiated specimens for $6\frac{3}{4}$ hours from a tube fed by a current of 180 kv. and 2 ma. Since the target-to-specimen distance was 24 cm. the intensity of the incident radiation must have been only about 1/64 as great as that employed in our experiments. We found that an irradiation time of 30 minutes was necessary to bring about an appreciable reduction of the sugar-lowering activity of insulin. Under the conditions of the experiments of den Hoed *et al.* an irradiation time of 32 hours would be required to produce a comparable effect with radiation of the same wave-length. It is apparent then that the negative results obtained in earlier experiments are due, in large part, to insufficient radiation [*cf.* Nitzescu, 1924].

Inactivation by ultra-violet radiation has been reported by several investigators [den Hoed *et al.*, 1929; Kuhn *et al.*, 1931; Freudenberg *et al.*, 1930; Ellis, 1925; Freudenberg and Eyer, 1932]. Maximum absorption and inactivation are obtained by radiation of wave-length 2750 Å. [Kuhn *et al.*, 1931; Freudenberg and Eyer, 1932]. This corresponds with the principal absorption band of tyrosine and cystine and suggests that either or both of these amino-acids are so-called "active centres." But since the absorption of X-rays is probably atomic rather than molecular, there is little likelihood of the same mechanism operating in our experiments. It would be unprofitable, therefore, without many more data, to attempt an explanation of the mode of X-ray inactivation.

SUMMARY.

1. The inactivation of insulin by acid-alcohol and by soft X-rays has been studied.
2. In both cases the phosphate-lowering activity of insulin was the most resistant to destruction.
3. Massive doses of insulin inactivated by X-rays produced hyperglycaemia; about 50 % of the phosphate-lowering activity was retained.
4. It is concluded that insulin owes its multiple activity to a number of "active groups," "centres," or "units" which vary in stability towards inactivating agents.

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CCXXIV. THE EFFECTS OF INSULIN AND ADRENALINE ON THE AMINO-ACID CONTENT OF BLOOD.

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(Received July 19th, 1933.)

In previous work it has been shown that insulin, injected into the normal animal, reduces the amino-nitrogen content of the blood and tissues [Luck *et al.*, 1928; Kiech and Luck, 1928], the latter even though hypoglycaemia be prevented by the simultaneous administration of glucose [Luck and Spaulding, 1928-29]. The rate of urea formation is also increased. More and more we have come to believe that these effects upon protein metabolism are independent of the effects upon carbohydrate metabolism. In the present paper we wish to adduce additional evidence which leads to this conclusion.

EXPERIMENTS WITH CRYSTALLINE INSULIN.

First of all it became necessary to determine for a certainty whether the amino-acid-lowering activity of insulin, hitherto studied upon therapeutic preparations only, was possessed by the crystalline hormone. We were aware of the possibility of the amino-acid activity being due to associated impurities, in much the same way that the transitory hyperglycaemic action of amorphous insulin is probably due to some principle other than insulin itself [Bürger and Kramer, 1930; Geiling and de Lawder, 1930]. Professor du Vigneaud was good enough to send us a specimen of crystalline insulin with which we sought an answer to the question.

Seven rabbits, previously starved for 48 hours, were injected subcutaneously with a freshly-prepared solution of crystalline insulin (1 cc. per kg. of body weight of a solution containing 1 unit per cc.). Two blood samples of 2 cc. were drawn from each—the first a few minutes before injection, the second $3\frac{1}{2}$ hours after¹. Reducing sugar and amino-acid nitrogen were determined by the methods of Folin [1922; 1928; 1929]. The following values were obtained.

										Mean
Amino-acid-N	Before	8.2	8.2	8.6	8.1	9.0	9.1	8.9		8.6
mg. per 100 cc.	After	6.7	6.9	6.6	6.5	7.4	7.8	7.4		7.0
Sugar	Before	99	96	97	94	92	94	94		95
mg. per 100 cc.	After	59	38	44	47	59	58	58		52

¹ To avoid hypoamino-acidaemia due to haemorrhage only two blood samples were drawn. Five animals receiving injections of saline in place of insulin and bled at 0, $\frac{1}{2}$, $1\frac{1}{2}$, $2\frac{1}{2}$, $3\frac{1}{2}$ and $4\frac{1}{2}$ hours after injection showed decreases in blood-amino-nitrogen content of 1.3 mg./100 cc. (mean value). Seven other rabbits which were bled but twice (0 and $3\frac{1}{2}$ hours after injection) showed no decrease. In two insulinised rabbits with six bleedings decreases of 3.2 mg./100 cc. were observed. The effect was maximum after $3\frac{1}{2}$ hours. These results are sufficient to account for our adoption of the "two-sample" procedure.

In the next paper of this series many more experiments, some with a different preparation of crystalline insulin, will be reported. The results are identical with these. It is apparent that the hypoamino-acidaemia produced by ordinary insulin is a result of the hormone itself, not of impurities.

Prevention of hypoglycaemia by the simultaneous injection of adrenaline. It occurred to us that a simple method of preventing hypoglycaemia arising from insulin administrations would be by simultaneous injections of adrenaline. An opportunity would thereby be presented of studying the rôle of hypoglycaemia in insulin hypoamino-acidaemia.

In the first group of experiments rats were employed. Attention was given to the effect of balanced injections of insulin and adrenaline upon the amino-nitrogen content of liver and muscle. By way of introduction it should be mentioned that earlier experiments demonstrated to us that the lowering of the amino-nitrogen content of blood induced by insulin is accompanied by a simultaneous reduction in the amino-nitrogen content of liver and muscle. This is in harmony with the finding of Kiech and Luck [1928] that the amino-nitrogen content of the animal as a whole suffers reduction.

In the present experiments 72 animals, starved for 36–40 hours at 28°, were used. The optimum conditions for obtaining a balancing of insulin hypoglycaemia by means of adrenaline were first determined. Samples were collected from the tip of the tail at –15, 5, 30, 60, 90 and 120 minutes after the initial injection. By repeated trial it was found that satisfactory control of the hypoglycaemia was obtained by injecting simultaneously 5 units of insulin per kg. of body weight, and 0.38 mg. of adrenaline per kg. followed by a second dose of adrenaline 65 minutes later (0.250 mg. per kg.).

Blood-sugar content (mg./100 cc.).

Animal	–15 min.	5 mins.	30 mins.	60 mins.	90 mins.	120 mins.
21	91	99	121	112	106	96
22	86	106	119	124	115	119
23	101	109	134	112	108	102
24	96	129	166	143	123	102
25	—	88	118	116	108	92

In three other animals from each of which only one sample was collected (2 hours after the initial injection) blood-sugar values of 94, 111 and 82 mg./100 cc. were obtained. Three other animals, paired with these three, were similarly treated except that 0.9 % saline was injected instead of the adrenaline. Values of 40, 43 and 39 mg./100 cc. were obtained.

Amino-nitrogen determinations on liver and muscle. The animals, injected both with insulin and adrenaline as described, were killed 2 hours after the first injection. The liver and thigh muscles were promptly excised, frozen in liquid air, powdered and analysed for amino-nitrogen [Luck, 1928]. The mean values presented in Table I were obtained.

Table I. *Amino-nitrogen content of liver and muscle.*

	Liver		Muscle	
	Number of animals	Amino-N content per 100 g. liver mg.	Number of animals	Amino-N content per 100 g. muscle mg.
Saline controls	23	47.7	24	58.8
Insulin	15	44.9	19	54.7
Adrenaline	11	44.6	10	54.9
Insulin plus adrenaline	15	43.3	18	55.9

It follows that the reductions obtained in the amino-nitrogen content of liver and muscle by injection of insulin are not appreciably lessened, if at all, by the simultaneous injection of adrenaline. In fact, adrenaline alone was found to lower the amino-nitrogen content—a point of some importance to which we shall presently return.

The rats used in the experiments just described were analysed in pairs, so selected that the members of any given pair were of the same sex, closely similar in age and weight, and, when possible, of the same litter. The one member of the pair received either saline or insulin, while the other received either adrenaline or insulin *plus* adrenaline. Analysis of the data from such "paired" experiments led to the same conclusion. Thus we observed in muscle that although the amino-nitrogen content of the groups of saline controls varied from 57.6 to 60.2, the values obtained from the experimental animals paired with these were uniformly less. For example, six rats which received insulin had an average amino-nitrogen content in muscle of 53.0. The saline controls paired with these averaged 57.6. The decrease induced by insulin was 4.6 mg./100 g. The corresponding values for nine adrenaline animals and their saline controls were 54.7 and 58.2; net decrease 3.5. For six animals receiving insulin *plus* adrenaline the corresponding values were 56.8, 60.2 and 3.4.

The changes in the basal level of amino-nitrogen content, reflected in the shifting saline-control values, seem to be partly seasonal in nature. They are of greater magnitude in liver but may be satisfactorily controlled by adoption of the paired-animal method.

To obtain confirmatory data we approached the problem afresh by studying the effect of balanced injections of insulin and adrenaline on the amino-nitrogen content of the blood of rabbits. The animals were bled but once, $3\frac{1}{2}$ hours after the initial injection. The most satisfactory control of the hypoglycaemia was obtained by injecting simultaneously 1 unit of insulin per kg. of body weight and 0.075 mg. of adrenaline per kg. followed by two injections of adrenaline (0.05 mg. per kg.) after 65 minutes and 2 hours respectively. In two animals,

Table II. *The effect of balanced injections of insulin and adrenaline on the sugar and amino-nitrogen content of blood.*

Animal	Sugar mg. per 100 cc. blood		Amino-N mg. per 100 cc. blood	
	0	$3\frac{1}{2}$ hours	0	$3\frac{1}{2}$ hours
1 A	105	88	9.4	7.8
2 A	100	105	9.3	7.6
1 B	112	118	7.1	6.7
2 B	106	115	9.6	7.3
3 B	108	83	8.3	8.2
5 B	121	129	8.8	8.7
6 B	100	149	8.8	8.1
7 B	110	114	8.9	8.2
5 B	115	118	8.8	7.5
7 B	88	119	8.1	6.5
2 C	114	82	10.0	7.3
1 C	119	91	9.3	7.0
3 B	121	110	7.8	6.9
6 B	119	149	8.4	6.6
7 B	105	118	8.5	6.6
3 C	118	108	10.2	8.2
4 C	122	128	9.3	6.6
5 C	100	95	8.3	6.5
	110	112	8.8	7.3

so injected, five blood-sugar determinations were made during $3\frac{1}{2}$ hours following the initial injection. All the values fell between 99 and 118 mg./100 cc. Eighteen experiments were performed. Despite the absence of hypoglycaemia a definite fall in amino-nitrogen was observed, the decrease being 17 % as against a mean fall of 21 % resulting from insulin alone. The means for all experiments follow:

Amino-N	Before	8.8	Sugar	Before	110.2
mg. per 100 cc.	After	7.3	mg. per 100 cc.	After	112.2

The complete results are presented in Table II. They indicate that a persistent hypoamino-acidaemia is obtained even when hypoglycaemia has been prevented.

EXPERIMENTS WITH ADRENALINE ALONE.

Although the literature contains two papers [Loeper *et al.*, 1926; Putschkow and Krassnow, 1928] on the amino-nitrogen content of the blood of adrenalectomised animals and other communications on the effect of adrenaline upon various aspects of protein metabolism, the results reported are markedly discordant. Since no one seems to have studied the effect of adrenaline upon the amino-nitrogen content of the blood of normal animals, we undertook the experiments summarised in Table III. Fasting rabbits again were used. It will be seen that adrenaline is fully as effective as insulin in lowering the amino-nitrogen content of blood.

Table III. *The effect of adrenaline on the amino-nitrogen content of blood.*

Animal	Sugar mg. per 100 cc. blood		Amino-N mg. per 100 cc. blood	
	0	$3\frac{1}{2}$ hours	0	$3\frac{1}{2}$ hours
1 A	116	238	10.1	8.5
2 A	114	263	9.8	7.5
3 B	104	256	8.6	7.7
2 B	102	250	9.3	7.2
5 B	111	298	9.2	7.8
7 B	110	282	9.6	7.5
6 B	—	254	9.0	7.0
8 B	111	200	8.6	6.8
	110	255	9.3	7.5

EXPERIMENTS WITH POSTERIOR PITUITARY EXTRACTS AND INSULIN.

The known antagonism between certain pituitary principles and insulin [Burn, 1923; Olmsted and Logan, 1923] led us to investigate the effect of posterior pituitary extracts upon insulin hypoamino-acidaemia.

In the first group of experiments extracts of Armour's powdered posterior pituitary were used. The extracts were prepared according to the U.S. Pharmacopoeia [1926] and were based on the acetic acid extraction method of Aldrich [1908]. In the remaining experiments the oxytocic and vasopressor preparations of Kamm *et al* [1928] known as pitocin and pitressin were used¹. Fasting rabbits were employed throughout. The results, presented in Table IV, show that pitocin with insulin caused very substantial decreases in the amino-nitrogen content of blood, the decreases being somewhat greater than we generally observe with insulin alone. At the same time the hypoglycaemic action of the insulin was reduced, especially with the smaller dose of pitocin (12 units/kg.).

¹ We are indebted to Parke, Davis and Company for supplying the pitocin and pitressin used in this work.

Table IV. *The effect of posterior pituitary extracts and insulin on the sugar and amino-nitrogen contents of blood.*

Sugar mg. per 100 cc. blood				Amino-N mg. per 100 cc. blood				Preparation used in conjunction with insulin (1 unit/kg.)
0	1 hour	2½ hours	3½ hours	0	1 hour	2½ hours	3½ hours	
117	75	49	52	8.6	8.3	8.3	8.2	Acetic acid extract 1 cc./kg.
98	73	51	56	9.7	9.4	8.5	9.0	
106	92	70	71	9.3	—	7.2	6.9	Acetic acid extract 2 cc./kg.
115	67	61	69	7.8	8.1	6.7	6.7	
125	112	89	99	8.1	7.2	6.4	6.2	Pitocin 12 units/kg.
101	133	95	80	9.2	8.8	7.3	7.0	
113	73	68	60	10.8	9.5	8.2	7.9	Pitocin 18 units/kg. (divided dose)
122	70	63	66	10.0	8.6	8.0	7.9	
110	80	67	69	8.0	8.7	6.8	6.8	Pitressin 10 units/kg.
100	117	—	65	8.1	8.5	6.8	6.8	
114	74	71	78	9.0	10.0	8.4	7.9	Pitressin 15 units/kg. (divided dose)
93	83	69	57	8.9	9.6	7.8	7.2	
110	118	69	65	9.1	9.6	8.1	7.9	Pitressin 20 units/kg. (divided dose)
114	123	61	71	8.8	9.1	8.8	8.5	
								" "

Pitressin given with insulin reduced somewhat the hypoglycaemic action of the latter with little if any effect upon its amino-nitrogen-lowering activity, with the curious exception, possibly, of an early but transitory increase in the amino-nitrogen content [*cf.* Bischoff and Long, 1931]. The anhydraemia usually caused by pitressin may account, partially, for this increase in amino-nitrogen. In one animal hypoamino-acidaemia was not observed.

The results with the acetic acid extracts are not easily interpreted. The larger dose of extract decreased somewhat the hypoglycaemic effect of insulin without altering its ability to lower amino-nitrogen. The smaller dose was without effect in lessening the hypoglycaemia but, oddly enough, was accompanied by but little hypoamino-acidaemia.

It should be mentioned that controls with insulin, pitressin and acetic acid extracts alone were run. Pitressin alone caused a transitory increase in amino-nitrogen similar to that obtained with insulin and pitressin together.

In harmony with the adrenaline experiments the results indicate that the amino-nitrogen-lowering activity of insulin is independent of its hypoglycaemic activity. However, the neutralisation of the hypoglycaemia by the pituitary preparations was complete in only two cases, so the results, though supporting the conclusions drawn from the adrenaline experiments, do not constitute a complete proof in themselves.

Incidentally the data on blood-sugar lend support to the conclusion of Magenta [1929] that pitocin is more active than pitressin as an antagonist to insulin.

DISCUSSION.

The principal result of these studies to which we wish to draw attention is the independence of the hypoamino-acidaemia induced by insulin of the characteristic effect upon carbohydrate metabolism—hypoglycaemia. An explanation, we feel, may be found in the experiments with adrenaline alone which caused decreases in amino-nitrogen content fully as great as those obtained with insulin alone. The fact that injected insulin provokes an increased secretion of

adrenaline [Cannon *et al.*, 1924; Houssay *et al.*, 1924; Kugelmann, 1931; La Barre and Houssa, 1932] leads us to suggest, in the light of our present findings, that adrenaline rather than insulin may be responsible for the decreases in amino-nitrogen reported in this series of papers. Other results obtained by the use of partially inactive insulin [Davis *et al.*, 1933] and low dosages of active insulin [Luck and Van Winkle] strongly support this conclusion. If adrenaline be the agent responsible for hypoamino-acidaemia, it is to be expected, as we have shown, that the prevention of insulin hypoglycaemia by adrenaline would be without effect upon the amino-nitrogen reduction. We are unaware of any work which indicates whether insulin would continue to provoke an increased secretion of adrenaline when hypoglycaemia is completely prevented by the simultaneous administration of glucose. Should it not do so, the observations of Luck and Spaulding [1928-29] could not be reconciled with the hypothesis that adrenaline, to the exclusion of insulin, is responsible for the hypoamino-acidaemia reported in this series of experiments. In consequence, the possibility that amino-acid lowering may be a primary and independent function of both insulin and adrenaline cannot be ignored. We hope that other experiments, to be undertaken before long, may give a conclusive answer to this question.

SUMMARY.

1. Crystalline insulin is as effective as amorphous insulin in reducing the amino-nitrogen content of the blood of normal rabbits.
2. The amino-nitrogen contents of liver and muscle are reduced alike by insulin, adrenaline, or balanced injections of insulin and adrenaline together.
3. Though hypoglycaemia be prevented by the use of balanced injections of insulin and adrenaline, hypoamino-acidaemia is still observed.
4. Adrenaline alone is as effective as insulin in reducing the concentration of blood-amino-nitrogen.
5. Pitocin lessens the hypoglycaemic action of insulin and increases slightly the amino-acid-lowering activity.
6. Pitressin given with insulin causes a slight and transitory hyperamino-acidaemia followed by hypoamino-acidaemia.

We are indebted to the Council on Pharmacy and Chemistry, American Medical Association, for supporting this investigation.

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CCXXV. STUDIES ON BACTERIAL PHOSPHATASES.

I. THE DECOMPOSITION OF PHOSPHORIC ACID ESTERS BY *CLOSTRIDIUM ACETOBUTYLICUM* WEIZMANN.

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(Received July 13th, 1933.)

THE conception of the phosphoric esterification of sugars as a necessary step in their metabolism in bacterial cells follows logically from considerations of carbohydrate breakdown in muscle and in yeast. But the experimental evidence in support of this conception in the case of bacteria is not very extensive, and information regarding the bacterial phosphatase systems which are responsible for synthesis and hydrolysis of phosphoric acid esters is very limited indeed. Virtanen and Tikka [1930] succeeded in isolating from a mixture containing, initially, glucose, inorganic phosphate and dried *Bacterium casei* ϵ two hexosephosphoric acid esters. One of these resembled the Robison ester, while the barium salt of the other had the formula $C_{12}H_{19}O_{16}PBA_2$. This appears to be the only reported case where the product of the phosphoric esterification of a sugar, as effected by bacteria, has been isolated. Virtanen had previously obtained some evidence of such a transformation with *Bacterium casei* ϵ [1924], *Streptococcus lactis* [1925, 1] and *Bacterium acidi propionici* *b* [1925, 2], but the ester was not isolated in any of these experiments. However it has been shown that several species of bacteria possess an enzyme system which is able to form methylglyoxal from hexosephosphates. Such species are *Bacillus delbrücki* [Neuberg and Kobel, 1929], *Bact. coli* [Fromageot, 1929], *Bact. lactis aerogene* [Neuberg and Scheuer, 1930], tubercle and timothy bacilli [Kuroya, 1931], *Cl. acetobutylicum* [Pett and Wynne, 1932, 1] and *Propionibacterium jensenii* [Pett and Wynne, 1932, 2]. These findings suggest that hexosephosphates play a significant part in the carbohydrate metabolism of these organisms.

Very little information is available in the literature with respect to the hydrolytic action of bacteria upon phosphoric esters. Manning [1927] has shown that *Bact. coli commune* ferments the sodium salts of both hexosediphosphoric acid and "hexosemonophosphoric" acid synthesised by yeast. Under either aerobic or anaerobic conditions decomposition proceeded with the formation of products normally found in glucose fermentations. The concentration of the diphosphate used was about 2.5 %. The fact that the ester is fermented by *Bact. coli* is not, in itself, evidence that a phosphatase is present, since it is conceivable that the glycolytic mechanism of the organism is able to ferment the glucose portion of the ester without preliminary hydrolysis. Recent observations of Boivin and Mesrobian [1933], however, have led to the conclusion

that a phosphatase is actually present in *Bact. coli*. In the present work it is shown that *Cl. acetobutylicum* possesses an active phosphatase, although sodium hexosediphosphate even in low concentration is not fermented by the living cells when no other carbohydrate is present.

EXPERIMENTAL.

In order to test the ability of the living organisms to ferment directly sodium hexosediphosphate, sterile solutions containing 1.2 % of the ester, 0.6 % of peptone, traces of NaCl and $MgSO_4$, and shredded filter-paper were inoculated with portions of suspensions of washed and of unwashed organisms removed from an active glucose-peptone culture at the age of 20 hours. The test media, contained in tubes, were incubated both aerobically and anaerobically at 37° and any gases formed in non-evacuated tubes were collected over dilute H_2SO_4 . Similar tubes containing 3 % glucose-0.6 % peptone were treated in the same manner. The glucose tubes all yielded normal volumes of mixed gases, but not even after 2 weeks' incubation did any tube containing hexosediphosphate evolve a trace of gas. In all cases, however, growth occurred, due to the presence of peptone. The results indicate that sodium hexosediphosphate in the relatively low concentration of 1.2 % is not fermented by the living organism in the manner in which glucose is fermented. No attempt was made to determine whether any hydrolysis of the ester took place. In view of Manning's results with *Bact. coli*, the fermentation of the same hexosediphosphate preparation by the latter organism was tested. Media similar to those described above were inoculated with *Bact. coli* prepared according to the method described by Manning. Small portions of a suspension of organisms added to sterile glucose-peptone and hexosediphosphate-peptone solutions caused vigorous fermentation in 24 hours. The sodium hexosediphosphate used in these experiments and in those which follow was prepared synthetically with the aid of zymin made from brewer's top yeast.

The hydrolysis of sodium hexosediphosphate by Cl. acetobutylicum in active glucose fermentations.

Into each of nine tubes containing shredded filter-paper were introduced 15 cc. of 5 % glucose-1.0 % peptone solution; the tubes were sterilised, and varying amounts of sterile sodium hexosediphosphate solution of known strength in addition to sufficient sterile water to make the volume 25 cc. were added. Each tube was inoculated with 0.5 cc. of an active maize culture of the organism and incubated for 5 days at 37.5°. The liberated gases were collected over dilute H_2SO_4 and the volumes, corrected to 20° and constant pressure, were measured at intervals during the fermentation and at the end. The final inorganic phosphate content of all tubes was estimated by the Briggs method, final reducing power by the method of Shaffer and Hartmann. Sodium hexosediphosphate allowed the fermentation to proceed only when present in low concentrations. When the degree of hydrolysis of the added ester was plotted against concentration of the ester as in Fig. 1 it was observed that greatest hydrolysis occurred when the ester was present in a concentration of approximately 0.05 %. But it is also apparent from Fig. 1 that with initial concentrations of the hexosediphosphate higher than about 0.2 % practically no additional inorganic phosphate was liberated. This may possibly be explained on the basis that the available enzyme was practically completely saturated by 0.2 % hexosediphosphate or it may be due to an inhibitory effect of the accumulated inorganic

phosphate. The added hexosediphosphate did not affect the rate of fermentation except in concentrations higher than 0.35 % where there was definite retardation.

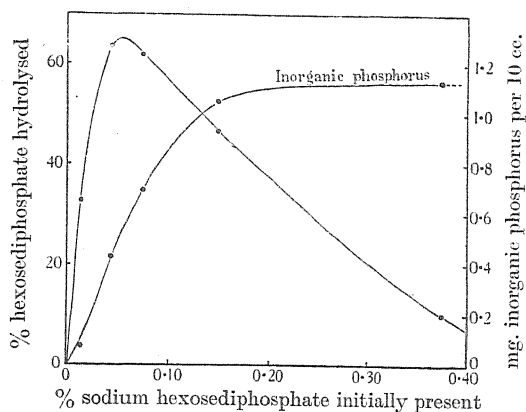


Fig. 1. Hydrolysis of sodium hexosediphosphate in active cultures of *Cl. acetobutylicum*.

Since it was shown in the last experiment that added sodium hexosediphosphate was partially hydrolysed in active glucose fermentations, the rate of hydrolysis in relation to the general progress of the fermentation as indicated by the development of acid was next studied. For this purpose four flasks were prepared, containing the solutions specified.

(1) Experimental flask: 300 cc. of a sterile solution containing 3 % glucose, 0.6 % peptone and 0.04 % sodium hexosediphosphate; (2) three control flasks: (a) 100 cc. of a sterile 0.04 % solution of sodium hexosediphosphate; (b) 100 cc. of a sterile 0.04 % solution of the diphosphate in which were dissolved quantities of acetic and butyric acids such that their concentrations approximated to the amounts present in a normal 3 % glucose culture at maximum acidity; (c) 300 cc. of sterile 3 % glucose-0.6 % peptone solution.

Flasks 1 and 2 c were inoculated with 5 cc. of an active maize culture and all flasks were incubated at 37°. The changes in the total acid and inorganic phosphate contents of flask 1 are illustrated in Fig. 2. No significant change in

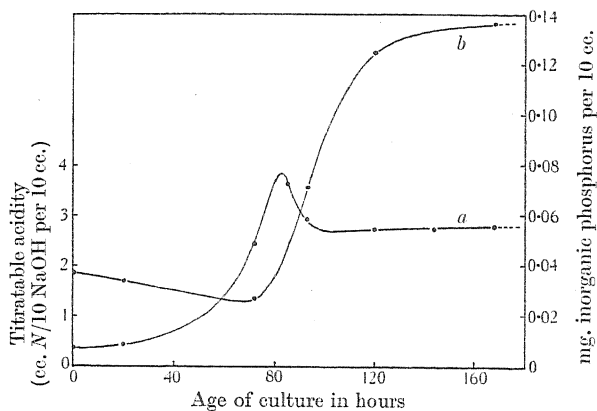


Fig. 2. Rate of hydrolysis of sodium hexosediphosphate in an active culture of *Cl. acetobutylicum*. a, titratable acidity; b, inorganic phosphorus.

inorganic phosphate occurred in any of the control flasks in 170 hours, but there was quite extensive hydrolysis of the hexosediphosphate in flask 1. The enzyme responsible for this hydrolysis did not come into effective action until the second phase of the fermentation. In this phase there is little or no further increase in the number of cells but frequently a decrease; during this period part of the accumulated acetic and butyric acids is converted into acetone and butyl alcohol at a rate which is comparable with the rate of accumulation of the acids in the first phase. The marked increase in the rate of hydrolysis in the second period suggests either that some of the cells have become more permeable to the enzyme or that the enzyme may have been liberated from cells which have actually undergone disintegration. It is not unlikely that a small portion of the liberated inorganic phosphate had its origin in the complex esters of the cell. The slight decrease in free phosphate in the early part of the fermentation suggests the formation of such esters during active growth.

Hydrolysis of glycerophosphate.

In the previous experiments the presence of a hexosephosphatase in active cultures was demonstrated: it was of interest to learn whether glycerophosphate, under similar conditions, would undergo hydrolysis. A series of flasks was prepared containing 3 % glucose-0.6 % peptone solution in which were dissolved varying amounts of sodium β -glycerophosphate (Boots); water was added to provide equal volumes in all flasks. The sterile media were inoculated with equal portions of an active maize culture of the organism and incubated at 37.5° for 5 days. From the amount of inorganic phosphate finally present in each flask the degree of hydrolysis of the glycerophosphate was calculated. The results are summarised in Table I. The amount of liberated phosphate increased with the concentration of glycerophosphate but the degree of hydrolysis calculated as % of added ester was low in all flasks, reaching a maximum when the concentration of the ester was approximately 0.02 %. Since glycerol is not fermented by this organism there can be no possibility that the accumulation of free phosphate is due to the operation of a glycerolytic mechanism which might conceivably release free phosphate by fermenting away the glycerol portion of the ester. It can be concluded, therefore, that a true phosphatase is present in the actively fermenting culture of the organism.

Table I. *Hydrolysis of sodium β -glycerophosphate in active cultures of Cl. acetobutylicum.*

Flask	Added glycerophosphate %	Total inorganic P after 5 days mg. per 10 cc.	Inorganic P derived from ester mg. per 10 cc.	Ester hydrolysed %
1	0.00	0.086	—	—
2	0.00	0.087	—	—
3	0.015	0.130	0.044	20.45
4	0.030	0.172	0.086	20.00
5	0.075	0.177	0.091	8.46
6	0.300	0.343	0.257	5.97
7	0.350	0.407	0.321	6.40
8	0.500	0.330	0.244	3.40
9	0.750	No fermentation		

Attempts to demonstrate synthesis of phosphoric esters.

Several attempts were made to demonstrate phosphoric esterification of glucose and fructose, in the presence of inorganic phosphate, by various preparations of the organisms. These included air-dried organisms, alcohol-ether-dried

organisms, dried organisms suspended in a small quantity of water and ground for several hours in an agate mortar with powdered glass and autolysed organisms. The results were all negative: in every case a definite increase rather than a decrease in inorganic phosphate was observed and, simultaneously, the reducing value of the mixture increased. Detailed consideration of these experiments is reserved until further studies have been made.

SUMMARY.

1. Sodium hexosediphosphate in 1.2 % concentration was not fermented by living *Clostridium acetobutylicum*. When present in 0.75 % concentration the diphosphate completely inhibited the fermentation of glucose in an otherwise favourable medium. A similar concentration of sodium β -glycerophosphate inhibited the fermentation of glucose.

2. In active glucose-peptone cultures of the organism both sodium hexosediphosphate and sodium β -glycerophosphate were appreciably hydrolysed. The presence of a true phosphatase in active cultures has been established.

3. The hydrolysis of sodium hexosediphosphate proceeded most rapidly during the second phase of the fermentation following the time at which maximum acidity was attained.

4. Attempts to demonstrate the synthetic activity of the phosphatase failed.

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CCXXVI. STUDIES ON BACTERIAL PHOSPHATASES.

II. THE PHOSPHATASES OF *CLOSTRIDIUM ACETO- BUTYLICUM* WEIZMANN AND *PROPIONIBACTERIUM JENSENII* VAN NIEL.

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(Received July 13th, 1933.)

INTRODUCTION.

In a previous paper [Heard and Wynne, 1933] the presence of a phosphatase in active glucose-peptone cultures of *Clostridium acetobutylicum* Weizmann was reported. It is the purpose of the present communication to describe some of the properties of this enzyme and of a similar enzyme present in *Propionibacterium jensenii* van Niel. Each of these organisms ferments various carbohydrates and certain related compounds. The first produces, as the chief end-products of glucose or starch breakdown, carbon dioxide, hydrogen, acetone, ethyl and butyl alcohols; the acetone and alcohols are derived from acetic and butyric acids formed as intermediary compounds. The second organism may be regarded as being representative of a rather large group of bacteria which ferment sugars and some related compounds with the formation of acetic and propionic acids [van Niel, 1928].

Phosphatases exhibit marked differences in their p_H -activity relationships. Those of mammalian tissues are, in most cases, much more active under alkaline than acid conditions, irrespective of the nature of the substrate. Usually the speed of hydrolysis reaches a maximum at about p_H 9, although, as Kay [1932] points out, this is probably only an apparent optimum since inactivation of the enzyme in more alkaline media may affect the exact identification of the optimum reaction. Roche [1931] observed that the phosphatase of certain mammalian erythrocytes appeared to differ from other mammalian phosphatases since it had a p_H optimum of 5.8 to 6.8 varying with the substrate. Among fungi and higher plants the few phosphatase systems which have been investigated are all most active under acid conditions. For example, the enzymes of *Aspergillus oryzae* and *A. niger* have p_H optima varying from 3.1 to 5.5 according to the method of preparation and the nature of the substrate [Asakawa, 1929]. Yeast phosphatase, whether acting hydrolytically [Kertesz, 1930] or synthetically [Euler and Nordlund, 1921], is most active at p_H 6.4, soya bean phosphatase at about p_H 5.3 [Kay and Lee, 1931] and rice phosphatase at p_H 4.3 [Horiuchi, 1931]. Regarding bacterial phosphatases very little is known. Virtanen [1927; Virtanen and Tikka, 1930] specified p_H 6.2 as the optimum for phosphoric esterification by *Bact. casei* ϵ . The fact that the phosphatase of *Cl. acetobutylicum* was found by Heard and Wynne [1933] to be active, hydrolytically, in growing cultures of the organism suggests that the enzyme prefers an acid medium. Quite recently it has been stated by Boivin and Mesrobian [1933] that both *Bacterium coli*

and *Staphylococcus aureus* possess two phosphatase systems which exhibit optimum activity at p_H 7 and 10. This conclusion was based on studies involving the autolytic liberation of free phosphate from the complex esters of the cell. No systematic study of the phosphatase of any bacterial species has come to the attention of the authors.

Regarding the specific behaviour of the various phosphatases no conclusion can yet be reached. Strong evidence was advanced by Kay [1928; 1932] in favour of the identity of the phosphatases of mammalian bone, intestinal mucosa, kidney and blood-plasma. Recent observations, however [Roche, 1931; Jacobsen, 1931; Edlbacher and Kutscher, 1932] indicate that certain other mammalian phosphatases exhibit marked differences of behaviour. Moreover, the fact that phosphatases of higher plants and fungi have p_H optima which are widely divergent from the values characteristic of the mammalian enzymes may possibly be regarded as evidence of certain fundamental differences in the enzyme systems. The phosphatases of the two bacterial species investigated in the present work are shown to differ not only from mammalian and plant phosphatases but also from each other in several respects.

EXPERIMENTAL.

(A) *Materials and methods.*

Substrates. The glycerophosphates used were "sodium glycerophosphate 644" prepared by the Eastman Kodak Company and shown by Kay and Lee [1931] to be almost entirely the β -ester, and sodium α -glycerophosphate (Boots) which contained only small amounts of the β -ester. Unless otherwise stated, the term glycerophosphate, wherever employed in this paper, refers to the β -ester. Sodium hexosediphosphate was prepared from commercial calcium hexosephosphate somewhat after the manner described by Neuberg and Sabatay [1925]. The preparation, as finally used, still contained a small quantity of a monophosphoric ester and a trace of inorganic phosphate. The sodium pyrophosphate employed was Baker's C.P. grade and was heated to remove traces of orthophosphate. All these compounds give alkaline solutions; they were therefore adjusted to the desired p_H with HCl before using.

General experimental conditions. The reaction mixtures were usually buffered with 0.025 *M* potassium hydrogen phthalate, this concentration being an effective buffer but having no appreciable inhibitory effect on the enzyme activity. Toluene was added, and the mixtures were rocked in L-shaped tubes in a water-bath at 37.5°. At suitable intervals 2 cc. samples were removed and added to 10 cc. portions of 5 % trichloroacetic acid solution in centrifuge-tubes. After centrifuging, 10 cc. of the clear liquid were removed with a pipette, and the inorganic phosphorus was determined by the Briggs method. Control tubes containing substrate + buffer and enzyme + buffer were included in all experiments. Phosphorus values are expressed as mg. of phosphorus present as inorganic phosphate in 10 cc. of reaction mixture.

Preparation of the enzyme. Attempts were made in the case of each organism to determine whether phosphatase was present in the culture medium in which the bacteria had been grown. When the number of cells had reached a maximum they were removed in the Sharples centrifuge, the clear liquid was concentrated at low temperature to a twentieth of the original volume and was tested for enzyme activity. No phosphatase could be detected in any such preparation: the treatment was probably not destructive since amylase was always present in the concentrates of the liquid obtained from the *Clostridium* cultures.

It was found, however, that in the case of both organisms phosphatase can be shown to be present in the dried cells as well as in suspensions of living cells to which toluene is added. Dried preparations were found to be more stable and more convenient and therefore they were employed in preference to preparations of toluene-treated organisms.

Cl. acetobutylicum was grown at 37° in a medium containing 4 % glucose, 1 % peptone and shredded filter-paper. After about 20 hours' incubation the culture was filtered through cheese cloth and centrifuged. The organisms were washed twice with distilled water, spread on a glass plate, dried at room temperature in a current of air, ground to a powder and preserved in a desiccator. This dry material loses about one-third of its phosphatase activity in a month at 4° and becomes practically inactive after 5 months. Some variations were observed in the activity of the dried organisms depending on the age of the culture at the time of the removal of the cells. Greatest activity per g. of dried cells usually occurs at the age of 18 to 20 hours: this period in the fermentation of carbohydrate by *Cl. acetobutylicum* is a critical one, for it is at this time that the cell count reaches its maximum, the acidity reaches its peak, and the second phase of the fermentation begins. Just after the period of maximum acidity the phosphatase activity per g. of dried cells usually drops appreciably, but it then rises again to a value approaching the maximum, and, after several hours, it finally falls to a value which may be only a small fraction of the maximum activity. This final drop is frequently very rapid in cultures more than 60 hours old. The physiological significance of these variations is not clear. It is possible partially to remove the enzyme from the dried cells by simple aqueous extraction. From such extracts and others, more concentrated and considerably purified preparations of the enzyme can be made. In the present work, however, only the dried cells were employed.

Propionibacterium jensenii was grown at 30° for 7 to 10 days in a medium containing 3 % glucose, or 2 % glycerol, and 0.6 % peptone. Sterilised extract of fresh starch-free yeast constituted 10 % of the liquid of the medium; the remainder was distilled water. The medium was adjusted to p_H 7.1 before final sterilisation. After incubation the organisms were removed by centrifuging and were treated in the manner described above. The phosphatase of the dried powder is quite stable: in 6 months in the desiccator at room temperature about one-half of the activity disappears.

In the experiments reported the dried organisms were used in the proportion of 0.10 g. to 10 cc. of reaction mixture, unless otherwise stated.

(B) *Some properties of the enzymes.*

(1) p_H -activity. Each tube of a series received 5 cc. of phthalate buffer having approximately the desired p_H except in the alkaline range where no buffer was used, 0.10 g. dried bacteria and 5 cc. of 0.3 % substrate. The p_H of each mixture was determined electrometrically at the beginning and end of each digestion and if it changed by more than 0.2 unit the experiment was repeated. Under these conditions the rate of hydrolysis is constant for the first 90 minutes, but during the next half-hour the velocity begins to fall off quite appreciably. It is usually preferable in such experiments to use initial velocities as criteria of activity rather than the changes brought about during an arbitrary time interval or at equilibrium. In the present work, however, the same optimum p_H is obtained whether initial velocities or equilibrium values are plotted.

Results with Cl. acetobutylicum. With sodium glycerophosphate, either α - or β -, the optimum is at approximately p_H 5.1, the β -ester being hydrolysed more rapidly than the α -form. The optimum for sodium hexosediphosphate is at p_H 6.0. Fig. 1 illustrates the result of a typical experiment in which glycerophosphate and hexosediphosphate were hydrolysed. The organism also possesses a very active pyrophosphatase whose p_H -activity relationships are illustrated in Fig. 2. The optimum is at p_H 7.2.

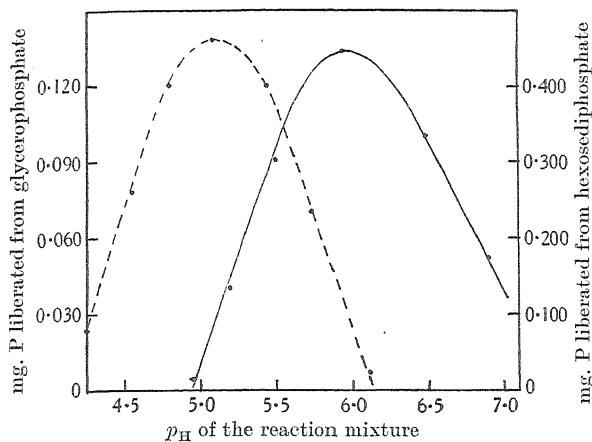


Fig. 1.

Fig. 1. The p_H -activity curves of the phosphatases of *Cl. acetobutylicum* acting on glycerophosphate (broken line), and on hexosediphosphate.

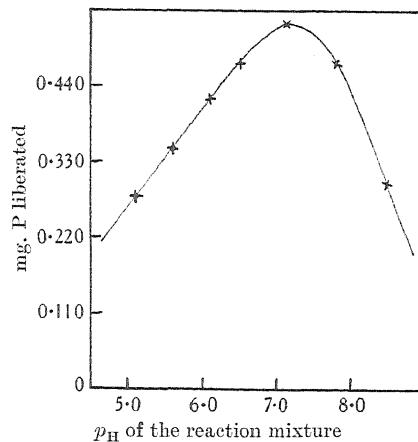


Fig. 2.

Fig. 2. The p_H -activity curve of the pyrophosphatase of *Cl. acetobutylicum*.

phosphate and hexosediphosphate were hydrolysed. The organism also possesses a very active pyrophosphatase whose p_H -activity relationships are illustrated in Fig. 2. The optimum is at p_H 7.2.

Results with P. jensenii. With sodium glycerophosphate, either α - or β -, the optimum is at about p_H 7.0, the α -ester in this case being hydrolysed faster than the β -form. With sodium hexosediphosphate the optimum is at about p_H 6.0 and with sodium pyrophosphate at p_H 7.0. The pyrophosphatase is here

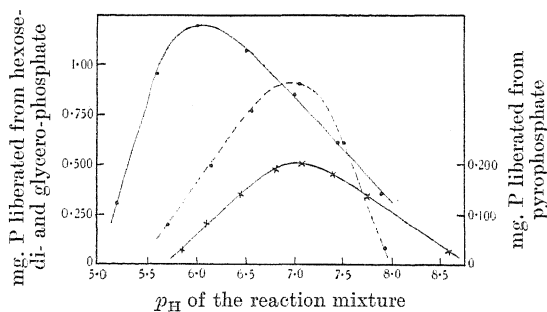


Fig. 3. The p_H -activity curves of the phosphatases of *Jensenii* acting on glycerophosphate (broken line), on hexosediphosphate and on pyrophosphate (with crosses).

very much weaker than the other two phosphatases whereas in the *Clostridium* it is much more active. Fig. 3 shows the results of a typical experiment in which the propionic acid organism was used.

(2) *Relation of reaction velocity to enzyme concentration.* In establishing a correlation between enzyme concentration and speed of reaction it is necessary to choose a concentration of substrate such that the largest amount of enzyme present in any digest is completely saturated. Furthermore, since progressive inactivation of the enzyme frequently occurs in all but the very early stages of the reaction, consideration must be limited to the linear portions of the progress curves such as those illustrated in Fig. 4. By making use of such constant initial

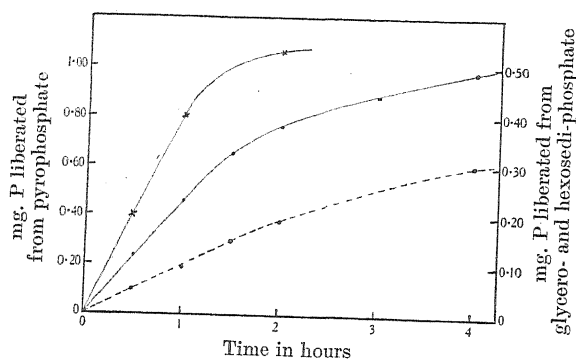


Fig. 4. Progress curves of the phosphatases of *Cl. acetobutylicum* acting on 0.15% glycerophosphate (broken line), on 0.15% hexosediphosphate, and on 0.5% pyrophosphate (with crosses).

velocities it was found that, with the bacterial phosphatases, strict proportionality between speed of reaction and amount of enzyme was readily demonstrated. For this purpose a series of digests was prepared, each tube containing 5 cc. of buffer at the optimum p_H , 5 cc. of 1% substrate, either glycerophosphate or hexosediphosphate, and increasing amounts of enzyme. In Fig. 5 is

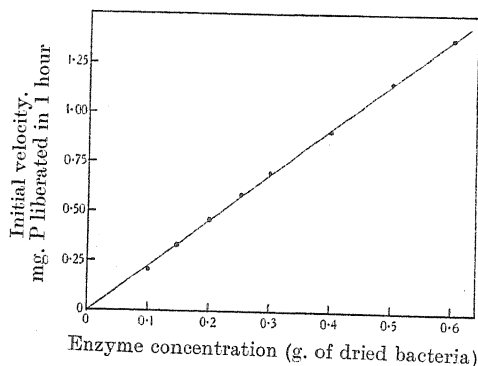


Fig. 5. The relation between enzyme concentration and rate of hydrolysis.

shown the result with the glycerophosphatase of the *Clostridium*, the speed of the reaction being expressed as mg. of phosphorus liberated as inorganic phosphate in 1 hour. The linearity is similar to that observed with other substrates and with the other enzyme preparation.

(3) *Effect of free phosphate.* Three solutions of NaH_2PO_4 adjusted to p_H 5.1 were prepared, of such strength that when 1 cc. portions of the solutions were diluted to 10 cc. the final concentrations of inorganic phosphate were those

listed in Table I. The inhibitory effects of these concentrations on phosphatase activity were determined in digests containing 5 cc. buffer, 0.1 g. dried organisms (*Clostridium*), 4 cc. of 0.4 % sodium glycerophosphate and 1 cc. of the phosphate solution. Control digests without added phosphate were run concurrently. The inhibitory effects, summarised in Table I, are of the same order of magnitude as those reported for mammalian phosphatase [Kay, 1928].

Table I. *The inhibition of phosphatase by inorganic phosphate.*

Initial concentration of phosphate	Degree of inhibition %
$M/500$	8
$M/200$	20
$M/100$	80

(4) *Effect of toluene.* Toluene was added in increasing amounts, up to 1 cc., to a series of tubes containing 0.1 g. dried organisms, 0.15 % substrate and suitable buffer in a total volume of 10 cc. Both organisms were used and both glycerophosphate and hexosediphosphate were employed as substrates but in none of the four experiments did the added toluene influence the rate or degree of the digestion. This result is at variance with the finding of Myrbäck and Euler [1929] who reported an inhibitory effect of toluene on the hexosephosphatase of dried yeast.

(5) *Effect of magnesium ions.* For the purpose of investigating the possible activating effect of magnesium ions the increase in inorganic phosphate in 1 hour was measured in a series of tubes each containing 0.1 g. dried organisms, 5 cc. buffer of the proper p_H , 4 cc. of substrate solution and 1 cc. of $MgCl_2$ solution of such strength as to give the desired final concentration. The concentrations of the three substrates, sodium glycerophosphate, sodium hexosediphosphate and sodium pyrophosphate, initially present in the digests, were 0.50, 0.18 and 0.50 % respectively. Suitable controls were run in all experi-

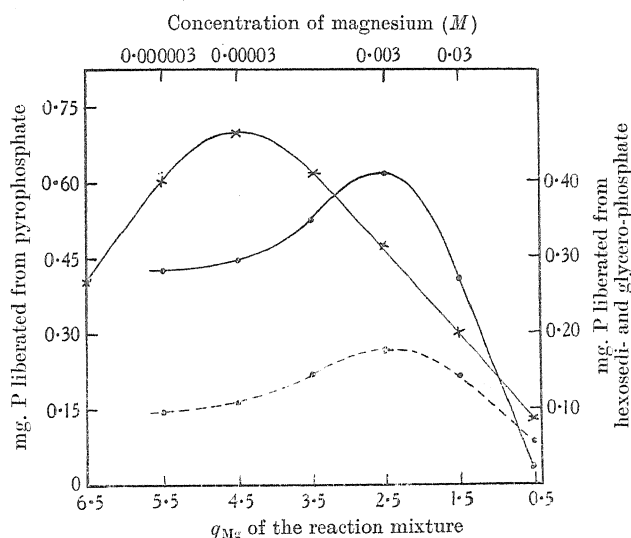


Fig. 6. The activity at different magnesium concentrations of the phosphatases of *Cl. acetobutylicum* acting on glycerophosphate (broken line), on hexosediphosphate and on pyrophosphate (with crosses).

ments. No difference could be detected between the action of MgCl_2 and MgSO_4 . No significant change in p_{H} was observed in any digest during the period of the experiment. The range of magnesium concentrations investigated extended from 0.3 to 0.000003 M . Since such a wide range of concentrations is difficult to plot conveniently, the practice of Jenner and Kay [1931] has been adopted: the symbol q_{Mg} has been used to represent the negative logarithm of the molar concentration of magnesium. This symbol has a meaning similar to that of p_{H} but avoids the question as to whether the magnesium is present entirely in the ionic form.

In Fig. 6 the effects of different concentrations of magnesium on the phosphatase activity of dried *Clostridium* are illustrated. For the hydrolysis of glycerophosphate and hexosediphosphate the optimum concentration is q_{Mg} 2.5 and for that of pyrophosphate q_{Mg} 4.5. In each case it is observed that a sufficiently high concentration of magnesium retards the action of the enzyme.

With the propionic acid organism much greater activating effects of magnesium were observed. Typical results are illustrated in Fig. 7 from which it is seen that the optimum concentration is q_{Mg} 2.5 for the hydrolysis of all three substrates.

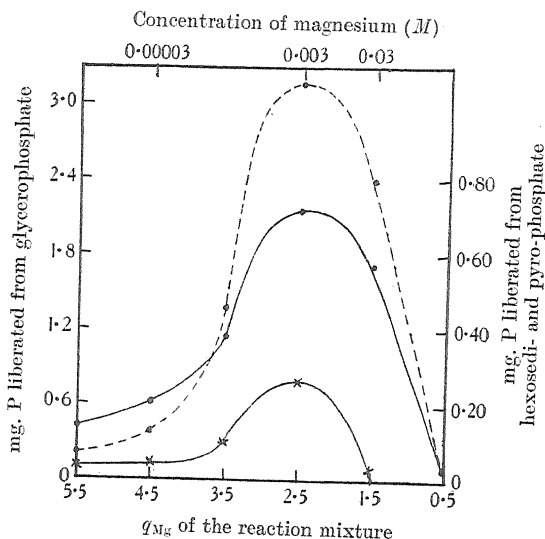


Fig. 7. The activity at different magnesium concentrations of the phosphatases of *P. jensenii* acting on glycerophosphate (broken line), on hexosediphosphate and on pyrophosphate (with crosses).

(6) *Effects of other ions.* In Table II are summarised the results of a study of the effects of several ions on the activity of the phosphatase systems of the two organisms. For purposes of comparison the value 100 is assigned to the activity in the absence of added ion and the relative activities as influenced by the various concentrations of ions are those listed in Table II. In the case of each substrate the same concentration was used throughout the series, namely 0.5 % sodium glycerophosphate, 0.18 % sodium hexosediphosphate and 0.5 % sodium pyrophosphate. The procedure adopted was the same as that described above for the study of the effect of magnesium, 1 hour's hydrolysis being chosen as a measure of activity except in the case of the hydrolysis of pyrophosphate by the propionic acid organism where 0.2 g. dried organisms was used and the

Table II. *Effect of various ions on phosphatase activity.*

Figures give the percentage hydrolysis under the influence of ions shown, as compared with the hydrolysis without added ions.

Ion (conc. of ion ...)	<i>Clostridium acetobutylicum</i>												<i>Propionibacterium Jensenii</i>											
	Glycerophosphate				Hexosediphosphate				Pyrophosphate				Glycerophosphate				Hexosediphosphate				Pyrophosphate			
	M/10	M/333	M/2000		M/10	M/333	M/2000		M/10	M/333	M/2000		M/10	M/333	M/2000		M/10	M/333	M/2000		M/10	M/333	M/2000	
Magnesium	88	175	150		51	148	125		82	150	200		500	1500	900		150	515	315		0	400	100	
Beryllium	0	50	92		0	49	87		0	89	100		0	23	40		0	10	50		—	—	—	
Calcium	77	89	95		98	99	100		50	100	99		30	55	90		25	60	90		20	70	94	
Zinc	80	86	96		60	74	94		0	100	140		30	35	69		22	48	68		0	100	160	
Strontium	46	56	100		34	81	94		0	90	103		20	30	90		18	29	80		—	—	—	
Cadmium	50	62	100		42	82	99		0	85	100		30	60	97		22	48	85		—	—	—	
Barium	40	90	95		60	79	89		0	66	95		98	99	96		90	98	101		—	—	—	
Mercuric	0	35	40		0	26	60		0	30	39		0	45	50		0	0	22		—	—	—	
Silver	0	50	80		0	10	50		1	59	95		0	0	35		0	0	50		—	—	—	
Potassium	99	98	100		102	100	100		97	98	100		94	96	98		105	100	100		—	—	—	
Sodium	100	100	101		100	99	99		95	97	100		99	99	100		98	99	100		99	98	100	
Lead	0	90	100		0	100	100		52	72	96		0	40	80		0	30	60		—	—	—	
Acetate	101	100	100		101	100	99		98	100	101		96	99	100		98	99	100		—	—	—	
Nitrate	100	102	100		102	100	100		85	90	100		100	102	100		92	100	100		—	—	—	
Sulphate	96	98	99		97	98	99		90	99	100		90	95	98		93	95	96		—	—	—	
Chloride	99	100	100		100	100	101		95	99	100		101	100	99		100	99	100		97	99	99	
Cyanide	M/100	M/500	M/2000		M/100	M/500	M/2000		M/100	M/500	M/2000		M/100	M/500	M/2000		M/100	M/500	M/2000		M/100	M/500	M/2000	
Fluoride	95	95	100		96	98	100		95	100	98		100	100	100		100	100	100		97	98	99	
	70	96	98		100	100	100		73	80	97		72	84	100		100	100	100		60	86	100	

digestion was allowed to proceed for 4 hours. This reaction is much slower than the hydrolysis of the other two substrates by this organism. The figures in Table II are comparable for each substrate and are reproducible to within about 5 % with a given enzyme preparation. Portions of the same enzyme preparation of each organism were used throughout.

An examination of Table II shows that magnesium was the only ion which accelerated the rate of hydrolysis of all substrates by the phosphatases of both organisms. Zinc, in low concentrations, caused definite activation of the pyrophosphatase of both organisms. Fluoride, in certain concentrations, retarded the hydrolysis of glycerophosphate and pyrophosphate but not that of hexose-diphosphate.

In conducting these experiments careful consideration was given to several important factors. The various solutions added to the digests were first adjusted to the p_H ultimately desired; the final p_H at the end of the experiment was determined in every case and, if a change greater than 0.3 unit occurred, the experiment was repeated. Secondly it was observed that in the presence of certain concentrations of some of the ions, particularly beryllium, barium, mercury, silver and lead, precipitation of the substrates occurred, a factor which may have led indirectly to retardation of the rate of hydrolysis. It is undoubtedly true also that the heavy metals have a direct inhibitory effect in certain concentrations. Finally it was necessary to consider the effect of each ion on the determination of inorganic phosphate from the points of view of the precipitation of the reagents and the development of the blue colour. Thus lead, silver and barium give such precipitates and were therefore removed with the proteins previous to the Briggs determination. Nitrate, cyanide and fluoride ions in 0.1 *M* concentration intensify the blue colour; a correction can be applied in the case of nitrates but not so accurately in the other cases. However, with cyanide and fluoride, no such disturbing influence was observed when the concentration was 0.01 *M* or lower.

DISCUSSION.

Points of interest arising from the present work are those concerned with (1) the question as to the specificity of magnesium activation and the possible rôle of magnesium as a coenzyme of phosphatase systems, (2) the question as to the specificity of the phosphatases themselves.

Of all the ions investigated, zinc is the only one other than magnesium which exhibited any activating influence in the concentrations employed¹. Zinc, it is true, appears to have a very limited, though quite definite, effect exerted only on the pyrophosphatase systems of both organisms; the degree of activation is of the same order of magnitude in the two cases and is confined to low concentrations of zinc. The limitation of the zinc effect to the pyrophosphatase in each case suggests that this enzyme differs in some manner from the hexosephosphatase and glycerophosphatase of the same organism. On the other hand the activation by zinc may be conditioned by the nature of the substrate rather than by that of the enzyme. The final interpretation of differences of this sort in relation to enzyme specificity must await further experimentation.

Magnesium, however, appears to stand apart from all other ions as an activator of all the phosphatase systems investigated in the present work. This result is in general accord with the findings of previous investigators who used

¹ Since these experiments were completed the arsenate ion has been found to cause a definite acceleration of the rate of hydrolysis of certain substrates by dried propionic acid organisms. An account of these observations will appear in a later paper.

mammalian phosphatases [Jenner and Kay, 1931]. The apparently specific response to magnesium of phosphatases in cells so different as those of mammalian tissues and bacteria, acting on various substrates, suggests that magnesium must play a fundamental rôle in the normal functional activity of these enzymes. It has been suggested that magnesium is a true coenzyme of the phosphatase system of mammalian tissues. In the case of the bacterial phosphatases studied in this work, this is rather unlikely: dried cells of *Cl. acetobutylicum* free from detectable magnesium were found to possess very appreciable phosphatase activity. For the purpose of testing this point the organisms were grown in a medium containing no magnesium other than that in the small amount of peptone present. 1 g. of the dried organisms was carefully ashed and the residue was examined for magnesium by a spot test, using *p*-nitrobenzoylazonaphthol [Feigl, 1931]. This test, sensitive to 0.0002 mg. of magnesium, gave negative results; the amount of magnesium present in 0.1 g. of the dried cells must indeed be negligible. In spite of this, when acting on glycerophosphate, 0.15 % in a total volume of 10 cc., the phosphatase present in 0.1 g. of the dried cells liberated from the ester 0.194 mg. of inorganic phosphorus in 2 hours. Obviously the enzyme did not require for its activity the presence of more than an infinitesimal amount of magnesium; it would therefore be incorrect to designate the magnesium ion as a coenzyme in the system under investigation.

Table III. *Comparative rates of hydrolysis of different substrates by bacterial phosphatases.*

Organism	Sodium glycerophosphate	Sodium hexosediphosphate	Sodium pyrophosphate
<i>Clostridium acetobutylicum</i>	1.0	2.0 \pm 0.4	6.0 \pm 0.6
<i>Propionibacterium jensenii</i>	4.5 \pm 0.5	8.0 \pm 0.2	0.4 \pm 0.3

The experiments have also a bearing on the subject of the specificity of the phosphatases, though it is realised that a study of the enzymes of only two bacterial species does not justify any wide generalisation. Points of difference and of similarity in the respective systems of the two organisms are apparent. For example the propionic acid organism has a pyrophosphatase which is very much weaker than the other phosphatases, whereas with the other organism the reverse is true. This difference and some other relationships are illustrated in Table III. In this table are summarised data based upon experiments in all of which the concentrations of substrate employed were such as to make possible the complete saturation of the enzyme, the amount of the latter being constant throughout. It is apparent that the ratio of the rates of hydrolysis of the different substrates is not the same for the two enzyme preparations. There are other differences between the phosphatases of the two organisms. The p_H optima with sodium glycerophosphate as substrate are rather widely separated, p_H 7.0 for the enzyme of one organism and p_H 5.1 for that of the other. The propionic acid organism hydrolyses α -glycerophosphate more rapidly than the β -ester, whereas with the other organism the reverse is true. The phosphatases of the propionic acid organism are apparently much more extensively activated by magnesium than are those of the other organism, though this difference may be related to differences in the physical conditions of the crude enzyme-containing preparations which were used. In general however it can be said that the effects of the several ions on the activities of the enzymes are similar for the two organisms. The behaviour of the enzymes of the two species reveals other

similarities. The p_H optima for the hydrolysis of either hexosediphosphate or pyrophosphate are the same for the two organisms. Finally the peculiar effects of zinc and of fluoride ions are essentially the same for both bacteria.

From the point of view of the substrates themselves differences have been observed. Sodium fluoride in concentrations up to $0.01M$ had no retarding effect on the hydrolysis of hexosediphosphate, whereas in considerably lower concentrations it retarded the rate of hydrolysis of glycerophosphate and of pyrophosphate. The differences in p_H optima were found to be sufficiently great in certain instances to suggest some form of specificity; it is possible that the use of purified enzyme preparations would reduce the differences, but it is unlikely that such optima as p_H 5.1 and 7.0 for the hydrolysis of different substrates could be shifted to the same level by methods of enzyme purification. Finally it may be noted that the optimum concentration of magnesium, namely q_{Mg} 4.5, for the hydrolysis of pyrophosphate by the propionic acid organism differs rather sharply from the optimum value, q_{Mg} 2.5, which characterises all other combinations of enzyme and substrate studied in the present work. This latter value is also optimum for the activity of mammalian phosphatases [Jenner and Kay, 1931].

Since the results reported reveal both similarities and differences a final decision with respect to the possible specific behaviour of the hydrolytic systems investigated cannot, as yet, be reached.

SUMMARY.

1. The phosphatase systems of *Clostridium acetobutylicum* Weizmann and *Propionibacterium jensenii* van Niel have been investigated in a series of experiments in which sodium glycerophosphate, sodium hexosediphosphate and sodium pyrophosphate were employed as substrates.

2. Various properties and relationships of the enzymes have been established, including p_H optima, correlation between enzyme concentration and reaction velocity, the effects of free phosphate, of toluene and of magnesium and several other ions on the rate or extent of hydrolysis.

3. The significance of the observations has been discussed in relation to (a) the questions of the specificity of magnesium activation and of the coenzymic nature of magnesium ions and (b) the problem of the specificity of the enzymes.

The authors take this opportunity of expressing their gratitude to Dr H. D. Kay for helpful discussion of several problems and to Dr C. B. van Niel for a culture of the propionic acid organism. Generous contributions of calcium hexosediphosphate from the Winthrop Chemical Company, Inc., of Windsor, Ontario have greatly facilitated this work.

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CCXXVII. THE DETERMINATION OF INORGANIC PHOSPHATE IN THE PRESENCE OF ARSENIC.

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(Received July 13th, 1933.)

For the study of the effects of the arsenate ion on the activity of phosphatases an analytical procedure is necessary which will eliminate the usual interference of arsenate with the colorimetric determination of phosphate. The close chemical relationship of these ions and the need of avoiding hydrolysis of the residual phosphoric esters are factors which present serious difficulties in the solution of the problem in hand. None of the procedures described in the literature was found to be dependable, particularly with higher arsenate concentrations. A method has been evolved, therefore, which permits the accurate colorimetric determination of inorganic phosphate even in the presence of M arsenate.

EXPERIMENTAL.

Method of subtracting a correction for arsenate alone.

In the few reported studies of the effect of arsenate on phosphatases [Neuberg and Leibowitz, 1927; Mayer, 1928; Macfarlane, 1930; Lohmann, 1930; Braunschtein, 1931; Morgulis and Pinto, 1932] the methods employed usually involved the use of a correction, though in some instances it is not clearly indicated how the arsenate effect was avoided. This correction is applied by subtracting from the arsenate-phosphate colour value, a value obtained for the same arsenate concentration alone.

Under the conditions in this laboratory the use of such a correction proved satisfactory only in the presence of small amounts of arsenate, and provided also that the time of colour development in the arsenate solution was, at the time of reading, precisely the same as with the solution containing arsenate and phosphate. With concentrations of arsenate higher than $M/1000$ this correction no longer gave reliable results, as shown in Table I.

Table I. *Showing the amount of "phosphorus" found in solutions of known arsenate and phosphate concentrations by subtracting a correction for the colour developed by arsenate alone. 0.1 mg. P was present in each case.*

Arsenate conc. in original solution	mg. P found after colour developing		
	5 mins.	60 mins.	120 mins.
$M/10$	0.460	0.296	0.171
$M/100$	0.264	0.200	0.115
$M/250$	0.181	0.150	0.108
$M/500$	0.138	0.108	0.104
$M/750$	0.121	0.109	0.103
$M/1000$	0.099	0.101	0.099
$M/1250$	0.101	0.102	0.101

The unreliability of the correction in higher arsenate concentrations appears even when using Lohmann and Jendrassik's modification [1926] of the Fiske and Subbarow method [1925] which, it has been claimed [Braunstein, 1931], gives dependable results owing to rapid development of the blue colour at 37°. The arsenomolybdate blue colour takes much longer to reach a stable intensity and to become proportional to the amount of arsenic present than is usually realised. Furthermore the mixture of arsenate and phosphate develops a blue colour at a rate even faster than phosphate alone. However, Table I shows that if the colours are permitted to reach a stable state, a condition which may require 2 hours or more, the correction can be more accurately applied. There remains, of course, the great disadvantage of having in some cases to apply a correction equivalent to 20 or more times the amount of phosphorus present.

Reduction of arsenate to arsenite.

At this point it was observed that arsenite, in contrast with arsenate, does not give a blue colour with phosphorus reagents, using either the method of Fiske and Subbarow [1925] or the Briggs [1922] modification of Bell and Doisy's method. This suggested reducing the arsenate to arsenite before determining the phosphate; details of a method for doing this have been elaborated.

Since this method was devised somewhat similar ideas have been found in two recent papers. Tananaeff and Potschinok [1932] suggested a procedure using Na_2SO_3 and HNO_3 before adding the regular reagents. A trial of the method as outlined, proved it to be satisfactory for amounts of arsenate up to about $M/500$, but not more. Tschopp and Tschopp [1932] in a valuable discussion of the formation of various "molybdenum-blue" compounds indicated that the addition of 1 cc. of 20 % NaHSO_3 just before adding the phosphorus reagents would prevent the formation of the arsenomolybdate. It is probable, however, that unless some time were allowed for reduction this procedure would not be very effective.

The reduction of larger amounts of arsenate requires appreciable time, but is accelerated by higher temperatures. Temperatures from 20° to 60° were tried, and 50° was selected as the most suitable for rapid reduction, having due regard to possible hydrolysis of residual phosphoric esters and to the removal of sulphur dioxide from the solution.

Effect of extra bisulphite on the phosphate determination.

The presence of sodium bisulphite in the usual phosphorus reagents had suggested its use for reducing the arsenate, rather than that of something which would have to be removed, such as H_2S ; but it was necessary to determine the effects of extra bisulphite and of added acid on the accuracy of the determination. Table II shows the results obtained in the presence of varying amounts of sulphuric acid and of sodium bisulphite, using Fiske and Subbarow's method. Similar flasks were left at 50° for various times, as indicated, and the phosphate determinations were then made. Each 25 cc. volumetric flask had received 1 cc. of standard phosphate solution containing 0.1 mg. phosphorus, then the acid and the bisulphite; water was added to make a volume of about 10 cc. At the stated time the flasks were removed from the bath, the reagents were added, and the solution was diluted to the mark as usual. The conditions of the experiment permitted immediate reading of the colour intensity.

Comparing numbers 4 and 6 in Table II it is noted that an increase in the acid diminishes the recovery of phosphorus; a more striking effect is observed

Table II. *Showing the effect on the phosphate determination of varying amounts of 3N sulphuric acid and of sodium bisulphite, acting for different times at 50° before the addition of phosphorus reagents. 0.1 mg. P was present in each case.*

	cc. H ₂ SO ₄	g. NaHSO ₃	mg. P found		
			20 mins.	40 mins.	60 mins.
1	0	0	0.098	0.102	0.099
2	0.5	0.5	0.096	0.100	0.099
3	0.5	1.0	0.091	0.094	0.095
4	1.0	0.5	0.097	0.101	0.099
5	1.0	1.0	0.092	0.095	0.094
6	2.0	0.5	0.090	0.092	0.092
7	2.0	1.0	0.087	0.088	0.088

as the bisulphite is increased. It is therefore necessary to avoid large amounts of bisulphite. By calculation 0.1 g. of NaHSO₃ can produce enough SO₂ to reduce 20 cc. of *M*/10 arsenate. It was therefore decided that about 0.4 to 0.5 g. of bisulphite and 0.5 cc. of acid per flask would be safe to use and give a sufficient excess of SO₂. These quantities leave the bisulphite in excess because 0.5 cc. of 3N H₂SO₄ is equivalent to only 0.3 g. NaHSO₃; if, however, trichloroacetic acid is used to precipitate proteins it supplies the needed extra acidity. 1 cc. of 5 % trichloroacetic acid is equivalent to 0.03 g. NaHSO₃. Moreover, it is safe to increase the sulphuric acid to 1 cc. if desired.

The treatment with 0.5 cc. of 3N H₂SO₄ and 0.4 g. NaHSO₃ at 50° for 1 hour has no appreciable hydrolytic effect on 10 cc. of 2.5 % sodium β -glycerophosphate, and only very slight hydrolysis occurs with 10 cc. of 1.2 % sodium hexosediphosphate.

Table III shows the times necessary for the reduction of various concentrations of arsenate at 20° and at 50°, all in the presence of 0.1 mg. of phosphorus. When the recovered value approximates to 0.1 mg. of phosphorus, reduction is considered to be complete.

Table III. *Showing the colour, expressed as mg. of P, developed by 0.1 mg. P in the form of inorganic phosphate and varying amounts of arsenate after reduction had progressed for different periods of time, at 20° and at 50°. 0.1 mg. P was present in each case.*

Arsenate concentration in original solution	20°				50°			
	15 mins.	30 mins.	45 mins.	60 mins.	15 mins.	30 mins.	45 mins.	60 mins.
<i>M</i> /10	0.337	0.263	0.190	0.130	0.253	0.177	0.126	0.101
<i>M</i> /100	0.227	0.182	0.135	0.109	0.156	0.109	0.099	0.100
<i>M</i> /250	0.147	0.114	0.104	0.100	0.116	0.100	0.100	0.100
<i>M</i> /500	0.134	0.104	0.100	0.100	0.104	0.100	0.100	0.098
<i>M</i> /1000	0.112	0.100	0.101	0.099	0.098	0.099	0.100	0.098
<i>M</i> /2000	0.102	0.100	0.098	0.100	0.100	0.100	0.099	0.100
None	0.098	0.098	0.099	0.098	0.097	0.098	0.100	0.099

From Table III it will be noted that heating at 50° is distinctly advantageous, *M*/10 arsenate being reduced in 60 minutes; *M* arsenate was found to be completely reduced in 70 minutes. It is also noteworthy that the accuracy is not impaired by leaving the flasks at 50° for an hour, even though reduction may be complete in less time. The experiment shown in the table was performed with the 0.1 mg. phosphorus present in 2 cc. of the arsenate solution, but the

same result is obtained in about the same time if the phosphorus is present in 10 cc. of arsenate solution of the concentration indicated. Dilution up to 15 cc. does not noticeably affect the reduction.

Procedure.

The procedure used is, therefore, as follows. All or part of the trichloroacetic acid filtrate of the sample from an enzyme digest is transferred accurately to a 25 cc. volumetric flask as usual. To each flask are added 0.5 cc. of 3*N* H₂SO₄ and about 0.4 g. of NaHSO₃. The necessary bisulphite can be estimated with sufficient accuracy without weighing, after a little practice, and is introduced into the flask through a funnel. The flasks are then left in a water-bath at 50° ($\pm 1^\circ$), usually for one hour, after which they are removed, and the reagents are added for determining phosphate. The elevated temperature causes a more rapid development of the blue colour than usual, and readings may be made at once with Fiske and Subbarow's method, or in 15 minutes with Briggs's modification of Bell and Doisy's method. The standards should be developed for at least 3 minutes at 50° in order to ensure a stable colour intensity.

Using the procedure outlined, attempts were made to determine known quantities of inorganic phosphorus, each in the presence of different amounts of arsenate. Typical results are given in Table IV, where it is seen that the accuracy of the method falls within the usual limits of error for the determination of inorganic phosphate in the absence of arsenate or other interfering ions.

Table IV. *Showing the amounts of phosphorus found in mixtures of various amounts of arsenate and of phosphate, after reduction of the arsenate at 50°.*

mg. P present	mg. P found				
	After 30 minutes' reduction of arsenate				After 60 minutes' reduction of arsenate
	M/1000 As	M/500 As	M/250 As	M/100 As	M/10 As
0.050	0.049	0.048	0.048	0.050	0.050
0.200	0.199	0.198	0.197	0.200	0.201
0.300	0.296	0.296	0.300	0.300	0.306
0.400	0.400	0.400	0.400	0.400	0.396

With the aid of this method an investigation of the effects of arsenate on the activity of the phosphatases of bacteria, yeast, taka-diaxase and mammalian tissue has been commenced.

SUMMARY.

In colorimetric phosphate determinations arsenate interferes by developing a blue colour with the ordinary reagents. In order to study the effects of arsenate on phosphatase activity this interference must be prevented, without, at the same time, hydrolysing the residual phosphoric esters. No method previously described was found to be sufficiently accurate in the presence of the higher concentrations of arsenate. A satisfactory procedure has been devised involving reduction of the arsenate to arsenite, a form of arsenic which does not interfere with the phosphate determination.

This study has been carried out under the supervision of Prof. A. M. Wynne, to whom the author expresses his indebtedness.

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CCXXVIII. STUDIES IN AVIAN CARBOHYDRATE METABOLISM.

IV. FACTORS INFLUENCING THE MAINTENANCE OF RESPIRATION IN SURVIVING BRAIN TISSUE OF THE NORMAL PIGEON.

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(Received August 25th, 1933.)

It is characteristic of the pigeon's brain that the rate of respiration falls very rapidly during survival in Ringer-phosphate solution. The addition of glucose [Gavrilescu and Peters, 1931] and more so of lactate [Gavrilescu *et al.*, 1932] increases the uptake over periods of an hour; even in this case the maintenance of respiration claimed by a series of workers for mammalian brain is not seen [cf. Loebel, 1925; Meyerhof and Lohman, 1926; Warburg *et al.*, 1924; Holmes, 1930; Quastel and Wheatley², 1932]. During the course of study of the catatorulin action of vitamin B₁ concentrates [Passmore *et al.*, 1933], we were obliged to attend to this matter. The problem was to determine the nature of the factors responsible for the gradual fall in respiration in phosphate buffer solutions. Though CO₂-bicarbonate gives different results with some tissues, the advantages of phosphate for control of p_H and rapid technique are great, and the differences from CO₂-bicarbonate may be less fundamental than they appear. The finding that there is a direct parallelism between the behaviour to vitamin B₁ in Ringer-phosphate of the minced brain and the functional state of the living brain shows that the behaviour under our biochemical conditions is not too artificial to be of value [Meiklejohn *et al.*, 1932].

Fig. 1 shows the behaviour of minced pigeon's brain, the hourly rate being plotted against the time. This shows changes better than the accepted method of plotting total respiration and time. It will be seen that none of the substrates induces maintenance. This is not a question of concentration of substrate; Gavrilescu *et al.* [1932] showed that K_m for *dl*-lactate was 0.024 *M*, making the concentration used here (0.033 *M*) practically maximum. The addition of galactose has only slight effect, though it was found by Sherif and Holmes [1930] to prolong respiration in the nerve (only after 4 hours).

This behaviour was interpreted as a gradual inactivation of some essential enzyme system, until the observations upon cyanide (Fig. 1, curve for succinate + KCN) showed that a respiratory system could remain in abeyance and then return to an original level³. Further study has shown that the addition of certain phosphorus compounds has a marked influence upon maintenance of respiration, when lactate is also present.

¹ Senior Demy Magdalen College.

² Quastel and Wheatley's [1932] curves fall off quite quickly (*e.g.* pig brain).

³ This interesting recovery from cyanide deserves more study; the % inhibition noted is close to that of Dixon and Elliott [1929]. It probably indicates formation of aldehyde [cf. Wieland, 1932].

Lactate essential. The influence of lactate upon prolonging respiration of brain tissue as well as muscle tissue is well known, as is also the fact that the action of glucose is mainly due to the lactate formed from it. Fluoride, for instance, stops glucose respiration but not that of lactate [Holmes, 1930; Krebs, 1931]. Nevertheless lactic acid is still very generally regarded as a product of anaerobiosis, not essential *per se* in cell metabolism. The discovery that the "catatorulin" action of vitamin B₁ was generally maximum only when lactate was present supports strongly the hypothesis that this acid is essential for normal cell respiration.

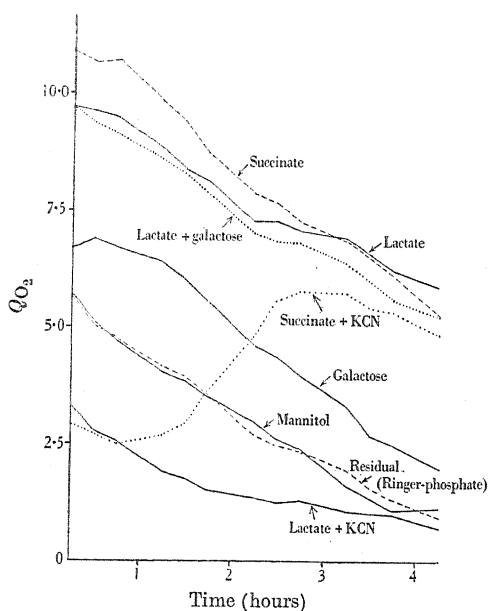


Fig. 1.

Fig. 1. Effect of various substrates upon respiration of minced cerebrum in Ringer-phosphate p_H 6.9. (Concentrations: lactate, 0.033 M ; succinate, 0.011 M ; galactose, 0.037 M ; mannitol, 0.015 M ; KCN 0.01 M .)

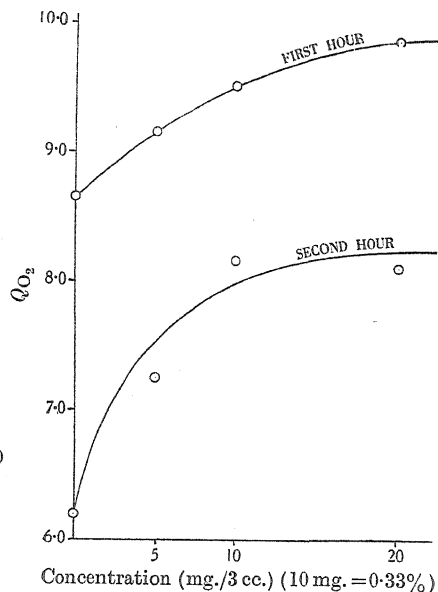


Fig. 2.

Fig. 2. Effect of concentration of candiolin upon respiration of minced brain in lactate, p_H 7.3.

It must be realised that no examination of a tissue has ever shown less than 10 mg./100 g. lactic acid; we may quote Davenport and Davenport [1928]; Fisher by use of iodoacetate in pigeon's tissues [1931]; Kinnersley and Peters in pigeon's brain [1930]; Owles [1930] found the minimum amount present in circulating blood to be 11 mg./100 cc. = 0.0012 M ; this may be concentrated at a surface to give 0.01 M in the tissue; comparison with the K_m curve referred to above, gives 0.024 M as concentration for maximum effect of the *dl*-acid.

If so, we can only reconstruct the normal respiration of the cell by adding to lactate other substrates. This kind of experiment has been done by others to study the "sparing" effect of one substrate upon another [Quastel and Wheatley, 1932], but not with the avowed purpose of using lactate as a basal "Ringer" metabolite.

It can be shown that addition of some substances to a basal Ringer containing lactate gives a much increased respiration, as compared with the respiration in presence of lactate or of the substrate alone. This was found to be the case with

crude Na hexosediphosphate (prepared from candiolin)¹. The increase is more marked in the 2nd hour than in the 1st, and is maximum when 10–20 mg./3.0 cc. (0.03 *M* approx.) are added to the solution (see Fig. 2).

Such an addition effect is of two possible types; it may either (*a*) be due to independent respiration with two substrates; in this case the respiration of lactate *plus* the other substrate will not exceed the sum of the independent respirations with lactate and substrate separately; or (*b*) the increased respiration may occur only when the lactate and substrate are together; it may be a definite interaction. Exp. 1 (Table I) was designed to test this point.

Table I. *Exp. 1.*

O₂ uptake mm.³/g./hr. for successive half-hour periods. 1000 mm.³ = $Q_{O_2} 5$.

Hours	...	$\frac{1}{2}$	1	1 $\frac{1}{2}$	2	
Ringer-phosphate*		950	800	650	550	
Ringer + candiolin		1145	1015	845	710	About 200 mm. ³ difference in each
Lactate only		1970	1750	1455	1275	
Lactate + Mg		1935	1750	1460	1280	No effect of magnesium
Lactate + pyruvate		2160	1920	1640	1500	
Lactate + candiolin		1995	1870	1660	1505	
Lactate + candiolin + pyruvate		1940	1820	1620	1480	
Lactate + candiolin + vitamin		1945	1840	1655	1505	

Conc. MgCl₂ 0.0016 *M*. Pyruvate 0.0038 *M*. Candiolin 0.33 %. Vitamin 1γ/3.0 cc.

* Calculated from a similar experiment.

The experiment is instructive; it shows increases by addition of pyruvate which equal that of candiolin; no increased effect with candiolin *plus* pyruvate, and as usual in the normal no change with vitamin addition². But since candiolin increases respiration with the Ringer-phosphate solution alone, there is no evidence that the candiolin interacts only in presence of lactate; the effect is of type *a*. The feeling that there was some factor at work influenced by lactate led to the trial of hexosediphosphate. Exp. 2 (Table II) shows that hexosediphosphate as purified from commercial candiolin does not even give maintenance effect. (The action of fluoride will be discussed later.)

We believe that the factor especially concerned is pyrophosphate, to which we were led by experiment upon the "catatorulin" effect (see a further paper).

Table II. *Exp. 2.*

(Calculated hourly.) mm.³/g./hr.

Hours	...	$\frac{1}{2}$	1	1 $\frac{1}{2}$	2
Ringer-phosphate		960	820	640	545
R.P. + hexosediphosphate		1030	940	805	635
Lactate		2140	1960	1735	1500
Lactate + hexosediphosphate		1980	1900	1795	1525
R.P. + fluoride		650	530	360	265
R.P. + hexosediphosphate + fluoride		670	575	390	280
Fluoride (0.008 <i>M</i>)		1480	1270	950	625
Lactate + hexosediphosphate + fluoride		1340	1200	920	630

Conc. of hexosediphosphate 0.008 *M*.

¹ The effect is much larger than that described by Quastel and Wheatley [1932] for the combination of succinate and lactate, though comparison is difficult as they worked with tissue previously autolysed for 3 hours.

² Sometimes vitamin addition shows a slight decrease in the normal.

Exp. 3 (partly shown in Fig. 3 and Table III) shows the effect especially well. It is supported by other experiments on the normal, which illustrate it in greater or less degree, and by the work upon the avitaminous brain. Here the action of pyrophosphate is additive only in presence of lactate¹.

Table III.

	mm. ³ /g./hr. 1000 mm. ³ = Q_{O_2} 5.						
Hours	...	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3
R.P.		1000	650	480	405	305	260
R.P. + pyrophosphate		810	530	470	380	320	350
L. + pyrophosphate + fluoride		1030*	(940)	530	490	390	310
L.		1960	1360*	1130	950*	790*	690*
L. + pyrophosphate		2070	1475	1390	1220	1210	1100
L. + pyrophosphate + Mg		2280	1860	1600	1510*	1430*	1280
L. + pyrophosphate + α -glycerophosphate		2655	2200	1900	(1880)	1710	1530
Same + Mg		2625	2100	2060*	1950*	1550	1400

* Bad duplicates.

L. = Lactate.

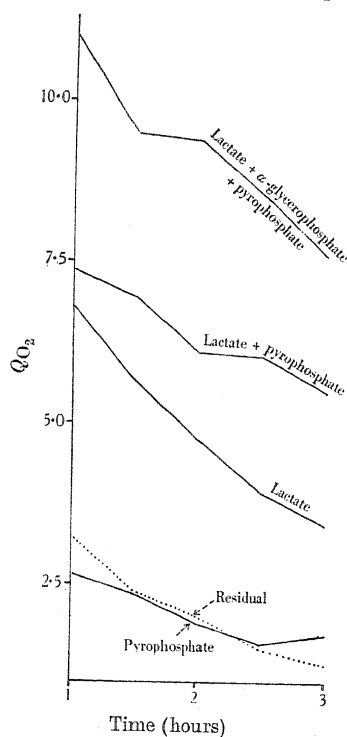


Fig. 3.

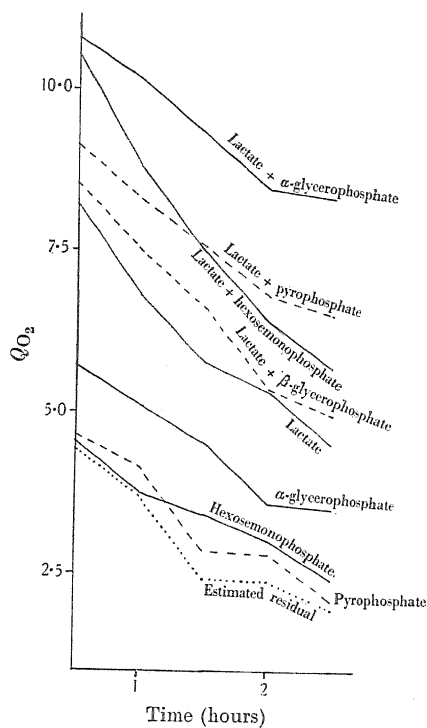


Fig. 4.

Fig. 3. Effect of pyrophosphate upon respiration of minced brain, p_H 7.3. (Concentrations: lactate, 0.033 M ; pyrophosphate, 0.0093 M ; α -glycerophosphate, 0.024 M .)

Fig. 4. Effect of various substrates upon respiration of minced brain p_H 7.3. (Concentrations: lactate, 0.033 M ; α -glycerophosphate, 0.024 M ; pyrophosphate, 0.019 M ; hexosemonophosphate, 0.013 M ; β -glycerophosphate, 0.023 M .)

¹ Controls with lactate + pyrophosphate and Ringer-phosphate solution with and without boiled brain show no oxygen uptake.

The additive action is an interaction of type *b*. So far this is the only case found and distinguishes the action of pyrophosphate from other active stimulants of respiration. It supports again the hypothesis that lactate is an essential component of the normal respiration system.

Exp. 4 (Fig. 4) shows the action of pyrophosphate; this and Exp. 5 (in Table V, also give the influence of α - and β -glycerophosphate and of Robison's hexose-monophosphate.

Davies and Quastel [1932] showed that bullock brain reduced methylene blue at about the same rates in presence of Na lactate and Na glycerophosphate, more slowly with succinate. Ashford and Holmes [1931] obtained twice the rate of respiration with lactate as compared with Na glycerophosphate.

We have found the β -glycerophosphate to be practically inactive. The effects obtained by others must have been due entirely to the α -glycerophosphate component of their preparations. This has a remarkable action, as can be seen by the abstract Table IV.

Table IV. *Exps. 4a and 4b.*

α -Glycerophosphate. (0.024 M) mm.³/g./hr.

Hours	...	(4a)			(4b)		
		1½	2	2½	1½	2	2½
Lactate only		1155	1070	900	1230	1140	890
α -Glycerophosphate only		900	720	705	920	850	610
L. + α -glycerophosphate		1810	1690	1665	1720	1620	1305
Ringer-phosphate only		—	—	—	475	400	330

Table V. *Exp. 5.*

mm.³/g./hr. 1000 mm.³ = Q_{O_2} 5.0.

Hours	...	½	1	1½	2	2½
R.P.		840	720	475	400	330
R.P. + α -glycerophosphate		1285	1100	920	850	610
R.P. + hexosemonophosphate		1140	900	650	550	440
L.		1955	1580	1230	1140	890
L. + α -glycerophosphate		2160	1980	1720	1620	1305
L. + hexosemonophosphate		2100	1670	1300	1160	900

The effect is largely independent of lactate, *i.e.*, it is of type α , and therefore differs from that of pyrophosphate in this respect. Exp. 5 shows that it has marked action apart from the lactate. As will be seen in a further paper, there is confirmation of this difference between the two substrates with the avitaminous brain, where vitamin interacts specifically with pyrophosphate but not with α -glycerophosphate.

Hexosemonophosphate promotes an increased oxygen uptake in the first hour, which soon drops either to the level of Ringer-phosphate or of lactate only, according to the constitution of the medium. It appears that there must be a rapid inactivation of the hexosephosphatase, but it is certain that this substrate (like hexosediphosphate) added to lactate alone induces no maintenance.

The finding that α -glycerophosphate is an independent additive substrate with lactate introduces at once the idea that we are dealing in brain with a system behaving like the new fermentation scheme of Embden *et al.* [1933], in which Meyerhof and McEachern [1933] concur. It is easy to understand that if free pyrophosphate is necessary in these experiments it may quickly diffuse from the cell and so rapidly fall to an ineffective concentration. The sudden

drop in metabolic rate would be then due to lowered concentration of essential substrates (lactate, pyrophosphate) for the energy metabolism, maintained by the cycle of carbohydrate change.

Exp. 6 (Fig. 5) and Exps. 7 and 8 in Table VI were designed to test whether a supply of several of the elements of Embden's fermentation scheme in presence of

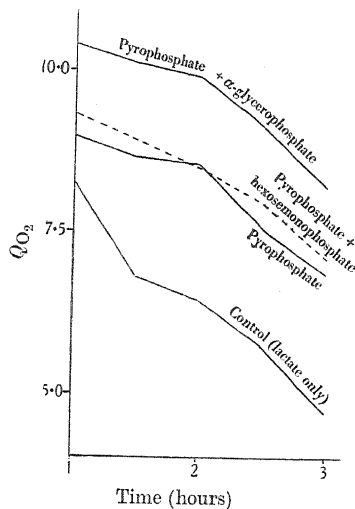


Fig. 5. Effect of combination of various substrates upon respiration of minced brain, in lactate, p_H 7.3. (Concentrations: lactate, 0.033 M ; pyrophosphate, 0.0093 M ; α -glycerophosphate, 0.024 M ; hexosemonophosphate, 0.0072 M .)

Table VI. *Exp. 7. Combined effect of substrates.*

Hours ...	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3
L.	1430	1100*	970	785	630	555
L. + α -glycerophosphate	2150	1450*	1130*	1070	1050	825
L. + hexosemonophosphate	1510	1220*	930	750	690	530
L. + pyrophosphate + α -glycerophosphate	2130	1900	1815	1410*	1290*	1140*
L. + pyrophosphate + α -glycerophosphate + fluoride	1555	1225	1010	900	750*	710
L. + pyrophosphate + hexosemonophosphate	1710	1430	1300	1160	1040	980
L. + pyrophosphate + α -glycerophosphate + hexosemonophosphate	2200	2000	1805	1700	1370	1400

Conc. α -glycerophosphate 0.024 M ; hexosemonophosphate 0.013 M ; pyrophosphate 0.0093 M ; fluoride 0.008 M .

* Mg.

Exp. 8. Cerebrum only.

Hours ...	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$
L.	2500	1970	1605	1350	1080*	990	790
L. + pyrophosphate	2455	2170	1940	1830*	1480*	1850*	1435
L. + α -glycerophosphate	2700	2330	2110	1880	1250*	1780*	1510
L. + pyrophosphate + α -glycerophosphate	2625	2405	2170	1985	1670*	1800	1670
L. + pyrophosphate + α -glycerophosphate + hexosemonophosphate	2665	2400	2170	1970	1900	1775	1600

Conc. as in Ex. 7.

lactate would give sustained respiration in the "brei"; Exp. 6 (Fig. 5) shows that addition of pyrophosphate to lactate remarkably improves maintenance of respiration, and that α -glycerophosphate further increases respiratory rate. The addition of hexosemonophosphate sometimes has no effect (Exps. 6, 8), or a slight one in the first hour only. Probably there is inactivation of the phosphatase present. Though as yet not proved, it is hard to believe that these substances are not those originally present in the tissue which cause so high an initial level of respiration. There is still more to be learnt, as Exp. 8 shows; the respiration in cerebrum only is still not properly maintained; but for these three experiments we have as average at the 2nd hour lactate alone 1142 mm.³/g./hr., + pyrophosphate and α -glycerophosphate 1791; and at 3rd hour lactate alone 828, with additions 1525, the remarkable average differences being 57 % and 84 % respectively.

Influence of fluoride. The presence of active fermentation following a scheme such as that of Embden and his colleagues in the respiring brain tissue can also be inferred from the effect of fluoride. This may be expected to influence any stage involving interaction with hexosephosphates. So, in Exp. 2, it is seen that some of the residual respiration is fluoride-sensitive and therefore due to hexosephosphate; this becomes some 5 % of the whole after 2 hours with added hexosephosphate. In presence of lactate, about one-third of the respiration is inhibited by fluoride; this is much more than even for Ringer-phosphate + hexosediphosphate. Hence, the improvement upon adding lactate is in part due to an increase in fluoride-sensitive respiration, the hexosephosphate (or triosephosphate) system.

In Exp. 7, fluoride reduced lactate + pyrophosphate + α -glycerophosphate to the level practically of lactate alone; all of this "extra respiration" is fluoride-sensitive. In Exp. 3, lactate + pyrophosphate is reduced to the level of Ringer-phosphate alone, again evidence of the interaction of the fluoride-sensitive system. The effect of α -glycerophosphate is to some extent independent of this: it is not fluoride-sensitive¹. According to Meyerhof and McEachern [1933] fluoride stops either the breakdown of triosephosphate to α -glycerophosphate and phosphoglycerate, or the breakdown of phosphoglycerate. So oxygen rather than pyruvic acid appears to be acting as the hydrogen acceptor.

The above experiments have been done at p_H 7.3. Change of p_H to 6.6 causes 30 % or more fall in the respiratory rate, see Table VII.

Table VII. *Effect of p_H on respiration rate (mm.³/g./hr.), 0-1 hour.*

Exp.	p_H 7.3	6.6	Decrease (%)
9	1730	1390	-20
10	1990	1491 (4)	-25
11	1990	1080	-46

(4) = average of 4.

Magnesium. In all the experiments except Exps. 8 and 3, Mg was added in some form, to avoid any possible complications induced by lowered concentration in the action of the adenylyl triphosphate coferment. In Exp. 3, tests with and without Mg do not show any consistent change. Since Lohmann [1931] found that the "lactic acid" coferment was not that of respiration, this is not surprising. In Exp. 1 $MgCl_2$ was added and in the others $\frac{1}{6}$ - $\frac{1}{15}$ vol. of Ringer-lactate, saturated with magnesium phosphate.

¹ This has been confirmed by experiments on the avitaminous brain.

DISCUSSION.

This work at present lacks the direct proof that the compounds added are the actual components of the tissue system; meanwhile, much may be done to help the study of this particular system by adding to Ringer-lactate pyrophosphate and α -glycerophosphate; 2-2½ hours of survival are so given. It seems unlikely that the effects are not general for brain tissue. The relation of pyrophosphate to active respiration may have a connection with the finding of Drury [1933] that pyrophosphate has stimulant effects upon the heart. For other tissues results may prove different. In phosphate buffers alone, Dixon and Elliott [1929] found that 0.033 *M* pyrophosphate inhibited markedly the respiration of muscle and liver tissue; there was a slight activation with 0.01 *M*. Engelhardt [1932] on the other hand inferred the participation of pyrophosphate from experiments with avian blood corpuscles showing the anaerobic splitting and aerobic synthesis of the easily hydrolysable phosphorus. Of the two paths of glycolytic change, one from glucose and one from glycogen, described by Ashford and Holmes [1929], it may be speculated that the precise function of that from glucose is to supply the essential component of the system, lactic acid; the other would then provide Embden's triosephosphates.

The case of pyrophosphate draws attention again to the necessity of bearing in mind possible concentration of diffusible constituents in the cell itself; this has been instanced recently by Jowett and Quastel [1933] for glyoxalase and glutathione. Comparison of tissue metabolism in different volumes may be dangerous near the critical margin for substances not strongly absorbed by the cell. A good quantitative illustration of this was given by Passmore *et al.* [1933] in the case of the critical level of residual tissue vitamin B₁. The optimum amount of pyrophosphate in the medium, 0.2 %, is of the same order as that possible in the cell, if we allow for some concentration at the active surface, from Lohmann's figures about 0.1 %.

EXPERIMENTAL.

Normal pigeons have been used throughout; they have been usually fed for 7-14 days upon the standard mixed laboratory diet. The technique of Passmore *et al.* [1933] has been used for the setting up of samples and obtaining oxygen uptake. 16 bottles can be filled from one mixed brain, making 8 duplicate estimations as a maximum. Values recorded are the mean of duplicates unless otherwise stated, calculated half-hourly.

Salts used. Na *dl*-lactate, made up in tubes from recrystallised zinc lactate and sterilised as before; 0.033 *M*.

Ringer-phosphate as before, but allowed to stand 2 days at *p*_H 7.3 before filtering.

Candiolin. "Ca hexosephosphate." Decomposed either by adding the required amount of Na oxalate, to the neutral suspension and allowing to stand, or by adding just sufficient HCl to dissolve and a solution of oxalate, and then immediately neutralising. The results did not differ. Tests showed that excess oxalate was not present.

Hexosediphosphate prepared as Ba salt from candiolin by Robison's method. Decomposed by Na sulphate.

Hexosemonophosphates. Specimen kindly supplied by Prof. R. Robison. Decomposed by Na₂SO₄ and standing some hours.

Ca α -glycerophosphate. Synthetic specimen supplied by Messrs Boots [King and Pyman, 1914]. Calcium removed by addition of oxalate; tests showed that excess oxalate was not present. Data supplied by Messrs Boots and Co. Loss at 140° 0.3 %. Residue on ignition 60.4 %. CaO 26.6 %. P₂O₅ 34.0 %.

Na β -glycerophosphate. Pure salt, supplied commercially (Messrs Boots).

Na pyrophosphate. Prepared by igniting Na_2HPO_4 (Sorensen). This gave no phosphate reaction with molybdate unless it had stood.

The concentrations of salts are those giving approximately maximum effects.

Temperature $38^\circ \pm 0.1$. p_{H} 7.3, unless otherwise stated. For experiments at p_{H} 6.6, the Ringer-phosphate prepared at p_{H} 7.3 was treated with HCl.

In making additions to the Ringer-lactate solution, the salts have been made up in 0.2–0.3 cc. of solution and so added to the bottles, making allowance for this by addition of less Ringer-lactate. The changes in concentration of lactate so produced would not influence results, but this has been controlled in the latest experiments by adding the necessary amount for lactate in 2.0 cc. Ringer-phosphate and making up to 3.0 cc. with the various additions. The small variations in tonicity have not been corrected. It was found by one of us with Dr Gavrilescu that very wide variations in strength of a glucose solution had little influence upon oxygen uptake. This is not so surprising when it is remembered that in a recognised surgical method, hypertonic solutions are injected into the blood to produce shrinkage of the brain.

SUMMARY.

1. The decrease in respiration rate in minced avian brain systems is substantially reduced in presence of lactate.
2. It is not reduced by galactose, mannitol, sodium β -glycerophosphate, hexosed- and hexosemono-phosphates. The last two substrates may influence the rate of fall initially.
3. Na pyrophosphate added alone has little influence upon maintenance of respiration in the surviving tissue. Added with lactate, it much increases the respiration rate, values considerably in excess of those with lactate alone being obtained. Lactate and pyrophosphate are essential constituents of the respiration system.
4. α -Glycerophosphate has a remarkable additional influence. Ringer-phosphate to which lactate, pyrophosphate and α -glycerophosphate have been added gives much better maintenance than lactate alone.
5. Experiments with fluoride indicate that survival respiration in lactate-phosphate solution involves in part a hexosephosphate stage.

We are indebted to the Medical Research Council and the Christopher Welch Trustees for grants in aid of the work. Also to R. W. Wakelin for skilful assistance. We are further grateful to Prof. Robison for a specimen of hexosemonophosphate.

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CCXXIX. DISTRIBUTION OF SUGAR AND NITROGENOUS SUBSTANCES IN WHEAT GRAIN¹.

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(Received August 30th, 1933.)

THE problem of the distribution of sugar and nitrogenous substances in wheat grain is of great practical and theoretical interest. A solution of this problem would provide the miller and baker with a number of indications concerning the composition of the different products of milled grain, enabling them to carry out the technological process upon more rational lines. The existing data on this subject are scanty.

Schulze and Frankfurt [1894] found that the germ of the wheat grain contains up to 25 % of sucrose on a dry weight basis. Apparently on the strength of this finding many subsequent investigators have considered that all the sugar of the wheat grain is concentrated in the germ. Having learned from a number of detailed analyses of wheat [Kiesel and Kretovitch, 1933] that in the whole ripe wheat grain the principal sugar consists of sucrose and that monosaccharides are almost absent, we have attempted to find out whether sucrose occurs in the endosperm as well as in the germ, and if so, whether the greater part of it is located in the outer or inner layers of the endosperm; also whether it can be found in the cells of the aleurone layer.

As regards nitrogenous substances we were interested principally in the question as to what portions are present in the cells of the aleurone layer, as it is known that flour from the latter fails to yield gluten.

We could not find in the literature data of direct analyses of the aleurone layer of wheat grain, although in the work of Teller and Teller [1932] on the nitrogenous substances of wheat bran it is indicated that the "epicarp" of the wheat grain contains little gliadin.

EXPERIMENTAL.

In order to determine the distribution of sugar between the germ and the other parts of the grain, a portion of the grain containing the germ was cut off from the rest and each part was analysed separately. As these analyses have shown (see below) that the portion of the grain without the germ² contained considerably more sucrose than the germ, it was desirable to obtain still more accurate determination of sugar distribution between the inner and outer layers of the endosperm and of the aleurone layer.

¹ The present investigation has been carried out in co-operation with S. S. Tanagos.

² Under the term "germ" we understand the portion of the grain called so by the millers, viz. the sprout.

For this purpose the grain was divided by a scalpel into three parts. First, the part containing the germ was cut off, and then the rest was divided into one part containing the inner kernel of the endosperm and another consisting of the bran coat with the aleurone layer and the outer layers of the endosperm.

Simultaneously with the analyses of the different parts of the grain an analysis of the whole grain was made as a check.

Material used for the investigation consisted of the grain "Cooperatoroka" of the 1931 crop from the Crimea. The analyses were carried out according to the method of Kiesel [1930]; in the aqueous solution obtained after extraction of the finely ground material by 82 % alcohol and evaporation of the latter *in vacuo* and after clearing of the solution by lead acetate the following determinations were made: (1) *reducing sugars*, (2) *sucrose*—by the difference between the sugar determination obtained after 5 minutes' hydrolysis by 2 % HCl at 70° and (1), and (3) *resistant polysaccharides*—by the difference between the sugar determination after 24 hours' hydrolysis by 1 % HCl at 70° and (2). All determinations were made according to the Bertrand method and calculated on a dry weight basis.

A summary of the results of analysis of the grain divided into two parts—one containing the germ and the other without it—and of the control analysis of the whole grain is given in Table I.

Table I. *Sugar as % of dry weight of different portions of wheat grain.*

Material investigated ...	Part containing germ; 22.24 % of weight of grain	Part without germ; 77.76 % of weight of grain	Whole grain (control)
Reducing sugars	0.21	0.0	0.0
Sucrose	2.18	1.45	1.67
Resistant polysaccharides	0.0	0.0	0.0

By control computation of the data of the first two columns on the whole grain basis the following was obtained:

Reducing sugars	0.04 %
Sucrose $1.13 + 0.49 =$	1.62 %
Total	1.66 %

Results of the investigation of the grain divided into three parts are given in Table II.

Table II. *Sugar as % of dry weight of different parts of wheat grain.*

Material investigated ...	A Part containing germ; 19.06 % of weight of grain	B Part without germ; 80.94 % of weight of grain	
		Outer layers of endosperm, with the bran coat and aleurone layer; 29.73 % of weight of "B"	Inner layers of endosperm; 70.27 % of weight of "B"
Reducing sugars	0.0	0.0	0.0
Sucrose	2.96	2.58	0.88
Resistant polysaccharides	0.01	0.0	0.0

Control computation of these figures on the whole grain basis shows the following ratio of the absolute quantities of sugar in the various parts of the

grain: 0.56 % (part containing germ) + 0.62 % (outer endosperm layer) + 0.50 (inner endosperm layer) = 1.68 %.

Material used for the investigation of the substances of the aleurone layer consisted of wheat grain, the germ and endosperm of which had been devoured by the storage weevil (*Calandra granaria* L.) by the ravages of which the inside of the grain had been cleaned out so neatly that the part left intact contained only the bran coat and the aleurone layer. Even under the microscope it was impossible to detect any particles of endosperm. Microscopic investigation showed that the cells of the aleurone layer and their contents had been left untouched by the weevil.

For the purpose of chemical investigation of the emptied grain it was subjected to a thorough grading and separation from foreign matter, ground as finely as possible and analysed for sugar content and various nitrogenous substances.

Repeated analyses have shown an absence of sugar from the empty grain and consequently from the aleurone layer.

Analyses of the nitrogenous substances contained in the empty grain, and consequently in the aleurone layer, have been carried out in the following manner.

A 10 g. air-dry aliquot of the material was extracted consecutively by water at 70–75° 4 times, 30 minutes each; by a 5 % solution of K_2SO_4 4 times, 3 hours each; by 70 % ethyl alcohol 4 times, 3 hours each; and lastly by 0.2 % solution of NaOH 4 times, 3 hours each.

Total nitrogen was determined by the Kjeldahl method in the original material, in the extracts obtained and in the residue.

Furthermore the aqueous extract was precipitated by normal lead acetate, and in the carefully washed precipitate a separate determination was made of the nitrogen of the water-soluble proteins; in the filtrate amide-N was determined by the Sachsse method and NH_3 -nitrogen by the Longy method. Results of the analyses are summarised in Table III.

Table III.

Fraction of nitrogen					% of dry weight	% of total nitrogen
Total	3.54	—
Non-protein	0.88	24.85
NH_3	0.33	9.33
Amide	0.22	6.20
N of water-soluble proteins	0.47	13.28
N dissolving in 5 % solution of K_2SO_4	0.18	5.09
N dissolving in 70 % alcohol	0.21	5.85
N dissolving in 0.2 % solution of NaOH	0.96	27.10
N insoluble in 0.2 % NaOH	0.80	22.60

SUMMARY.

The data obtained permit the following conclusions.

Sucrose is contained not only in the germ of the wheat grain but also in the endosperm, the total amount being greater in the latter than in the former.

Considering that the weight of 100 whole grains is 3.200 g. and the weight of 100 empty ones 0.371 g., i.e. that the latter amounts to 11.6 % of the weight of the former, and taking into consideration the data of Table II, it is easy to estimate that the concentration of sugar in the outer layers of the endosperm is about 4.8 times greater than in the inner layers. This fact shows that the sugar is distributed in the grain in the same manner as is gluten [Cobb, 1925].

Analyses of the grain emptied by the weevil show that there is apparently no sugar in the aleurone layer.

As regards nitrogenous substances the results obtained by us confirm the data of Teller and Teller [1932], who found that wheat bran contains little gliadin. Our results indicate that there is practically no gliadin in the cells of the aleurone layer. Thus it becomes clear why flour from the cells of the aleurone layer does not yield gluten.

As regards glutenin and albumin, they compose the principal part of the protein substances of the aleurone layer, and the aleurone grains of wheat apparently contain a very insignificant amount of globulin. It is possible that during the process of extraction a part of the globulins may have passed into the water-soluble protein fraction, as the empty grains contain 6.57 % ash of dry weight substance, but the error caused by this factor could not be very great as in a volume of 100 cc. of the extracted liquid the concentration of salt was approximately 0.6–0.7 %.

Thus the general conception of aleurone grains as deposits of globulins does not apply to the aleurone grains of wheat.

In regard to the conclusions drawn from analysis of the empty grains it might be argued that the weevil while eating the grain might have caused some changes in the substances of the aleurone layer and that therefore the picture given by our analyses is not quite true. We do not however regard this as a serious objection since the microscopic appearance of the aleurone layer of such emptied grains is perfectly normal.

In conclusion we wish to express our thanks to Prof. A. R. Kiesel for his interest in this work.

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CCXXX. SOME CELL-WALL CONSTITUENTS OF *CETRARIA ISLANDICA* ("ICELAND MOSS").

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(Received August 31st, 1933.)

CHEMICAL investigation of the lichen commonly known as "Iceland Moss" (*Cetraria islandica*) has extended over a considerable period of time, the earliest scientific examination of the material being recorded by Berzelius [1813]. In the main, most attention has been devoted to two groups of substances: (i) the so-called lichen acids and (ii) the water-soluble carbohydrate constituents (lichenin, *isolicheinin* etc.), which form the major part of the solids in the lichen. Concerning these latter a very large literature exists. The existence of a number of definite polysaccharides has been established and suggestions put forward for the constitution of certain of them. All appear to be derived from glucose, although Karrer and Joos [1924] reported the isolation of galactose and mannose from one of the fractions. The chemistry of these soluble carbohydrates has been studied so fully that in the present work no attempt has been made to investigate the subject further.

The lichen acids and the soluble polysaccharides comprise in all some 80 % of the total dry matter of the tissue. After their removal, there remains a residue, mainly carbohydrate in nature, consisting of cellulose, various hemicelluloses etc. It was this residue which formed the material for the present investigation. Hitherto it seems to have received little attention. Ulander and Tollens [1906] reported the presence of glucose, mannose and galactose therein, an observation confirmed by Hesse [1916]. Salkowski [1921], after hydrolysis of the water-extracted plant by dilute sulphuric acid obtained a resistant xylan-free ashless material, which he showed was not lignin. Poulsson [1906] reported the presence of 3 % of pentosan in the water-insoluble residue, basing his conclusion on a determination of furfuraldehyde. From the purely chemical standpoint, therefore, the present work was designed to furnish further information as to the chemical constituents of an already much-studied plant material. At the same time it was hoped that the results would make it possible to draw a comparison between the cell-wall components of this lichen and those of the higher plants, which have received so much attention in the past few years. The lichens differ so widely from the higher plants in their growth and habit, that it seemed of interest to discover how far the classes resembled one another in the chemical make-up of their cell-walls. It was found that, while there is a resemblance, in that cellulose and the hemicelluloses constitute a considerable proportion of the cell-wall of this particular lichen, the hemicelluloses are of a somewhat unusual type, apparently simpler in structure than those of the higher plants, in being free from pentose residues; while the pectins, of almost universal distribution in the cell-wall of the higher plants, are absent from this lichen. The possible significance of these differences is commented upon later.

EXPERIMENTAL.

1. *Preliminary examination of the lichen.*

In the first place a preliminary survey was made in order to estimate the relative amounts of fractions corresponding roughly to the different groups of carbohydrates present in the lichen. For each experiment, 100 g. of the oven-dried lichen, picked by hand free from extraneous material such as pine needles, were extracted with the following solvents, in the order given:

- (a) Cold water; 12 extractions, each of 24 hours.
- (b) Hot 95 % alcohol; 12 extractions, each of 6 hours.
- (c) Hot water (90°–95°); 20 extractions, each of 6 hours.
- (d) 4 % sodium hydroxide; 3 extractions at room temperature, each of 4 days, followed by 1 extraction at 80° for 6 hours.

At the end of this treatment, the residue was washed with dilute acetic acid until free from alkali, then with hot water and dried. The substances extracted were obtained in solid form, in the first three cases by direct evaporation on the water-bath, and in the case of fraction (d) (corresponding to the hemicelluloses) by precipitation with acetic acid and alcohol.

In the case of the hot water extractions, and to a less extent with the alkali, great difficulty was experienced in handling the material, on account of its extremely gelatinous nature, which made some mechanical loss unavoidable.

Estimations of furfuraldehyde and carbon dioxide yields were made on all the fractions, as well as on the original lichen. The carbon dioxide yields from all fractions were treated as if they arose entirely from uronic anhydride, except in the case of the alcohol extract, which contained the lichen acids—substances which might be expected to yield some amount of carbon dioxide on distillation with 12 % hydrochloric acid. The results of this preliminary examination of the lichen tissue are summarised in Table I.

Table I.

Fraction	Dry weight as % of lichen	Furfuraldehyde yield %	CO ₂ %	Uronic anhydride %
Original lichen	—	1.48	1.65	—
Cold water extract	5.71	0.66	0.95	3.80
Alcohol extract	8.54	Trace	4.72	—
Hot water extract	64.20	1.29	0.85	3.40
NaOH extract	3.20	1.68	1.27	5.08
Residue	10.80	1.52	1.40	5.60

The results indicated that the fractions which it was proposed to study in detail (*i.e.* the "hemicellulose" and cell-residue) contained considerable amounts of furfuraldehyde- and carbon dioxide-yielding constituents, presumably of a "polyuronic" nature. The cell-residue, in fact, contained more of these substances than the hemicellulose fraction, indicating either a partial oxidation of cellulose units or the presence of substances of a "cellulosan" type resistant to extraction by the 4 % alkali usually employed.

It may be noted that the water-soluble fractions (lichenin and isolichenin mainly) gave appreciable amounts of carbon dioxide, and thus appeared to contain uronic anhydride units, a fact which seems to have escaped notice hitherto. (It may be recalled, however, that Ulander and Tollens [1906] reported the presence of traces of pentosan in the lichenin fraction, based upon furfuraldehyde determination; since the uronic anhydrides themselves give

about 16 % of furfuraldehyde on distillation with HCl, it seems possible that the pentosan of Ulander and Tollens may have represented uronic anhydride units. The figures in Table I show that, in the cold water extract, the whole of the furfuraldehyde could be accounted for as derived from uronic anhydride; in the hot water extract about 0.5 % of furfuraldehyde cannot be so accounted for.)

2. Attempted isolation of a pectin fraction.

The preliminary analysis had shown that the amount of furfuraldehyde-yielding bodies in the lichen was small, none of the fractions giving so much as 2 % furfuraldehyde. Thus pectic substances were not expected to be present to any great extent; in view of their possible presence, however, several attempts were made to isolate a pectin fraction. Extractions were carried out on the water-extracted residue with hot 0.5 % ammonium oxalate and the extracts precipitated with alcohol. After redissolving in water and reprecipitating with alcohol the rather gelatinous material was again dissolved in water, and, in aliquot parts of the solution, pectin was estimated by the calcium pectate method. Only a very small amount of an acetic acid-insoluble calcium compound was obtained, and this bore no resemblance to calcium pectate as usually prepared. Further, the calcium content of the substance was found to be 16 % and its carbon dioxide yield 11.3 %, whereas calcium pectate contains 7.4 % Ca and gives 17.6 % CO₂. Two estimations gave a yield respectively of the calcium compound, 0.14 % and 0.42 % of the dry tissue after water extraction. Other experiments on similar lines failed to show the least trace of pectic substance, either in the tissue residue or in the water extracts, and the conclusion was therefore drawn that the lichen contained no pectic substances. The calcium compound obtained was probably the salt of one of the lichen acids.

3. Extraction of hemicelluloses.

The hemicellulose fraction was obtained by extraction of the cell-residue (after complete removal of the water- and alcohol-soluble material) with 4 % sodium hydroxide. The residue was treated, in portions of 200–300 g., with 4 litres of 4 % alkali, twice. The clear extracts, filtered through calico, were then made slightly acid with acetic acid and allowed to stand for some hours. Only in one case was a slight turbidity produced, but not enough solid separated to be removable by filtration or by centrifuging. A hemicellulose of the "A" type thus appeared to be absent.

To the faintly acid solution a half-volume of acetone was added, a copious precipitate being produced; after removal of this, the addition of excess acetone to the filtrate gave no further precipitate, so that the only hemicelluloses found in the sodium hydroxide extract were those of the "B" type [Norris and Preece, 1930]. This fraction was further separated by means of the copper method of Norris and Preece [1930] into fractions B1 and B2. Both were obtained in the form of almost white powders, of low ash content, quite stable in air and not darkening when heated to 100° for 4 hours. The relative yields obtained in different extractions varied somewhat, as shown in Table II.

Table II.

Extraction No.	Weight of lichen residue taken g.	Yield of hemicellulose	
		B 1	B 2
		g.	g.
1 a	184	12.0	5.1
1 b	203	16.5	8.8
2	300	31.0	5.0

4. Isolation of a further hemicellulose.

It was noted, in the small-scale extractions, that a considerable amount of furfuraldehyde- and carbon dioxide-yielding material appeared to resist extraction with 4 % alkali (Table I). Examination of the corresponding residues after the large-scale preparation of hemicelluloses B1 and B2 confirmed this observation; further, the material on acid hydrolysis was found to give as much as 12 % mannose (estimated as the phenylhydrazone) and 5 % galactose (estimated as mucic acid). Evidently therefore polysaccharides other than cellulose were still present in this residue. Such resistant "cellulosans" are of course frequently met with in plant tissues, xylose being possibly the sugar most commonly concerned.

An attempt was made to isolate this more firmly combined hemicellulose by extraction with 17 % sodium hydroxide, the extraction being allowed to proceed for several days at room temperature. From the alkaline extract, no precipitate was obtained on acidification, but on subsequent addition of alcohol a bulky yellow-brown precipitate appeared. This was redissolved in 2 % sodium hydroxide and precipitated as a copper compound by addition of an equal volume of Fehling's solution; no further precipitation occurred on addition of acetone. The copper compound was decomposed with dilute acid and the hemicellulose obtained in the usual manner, the yield of the product varying from 7 to 11 % of the alkali-extracted residue taken (*i.e.* approximately 1 % of the original lichen).

5. Examination of the individual hemicelluloses.

Hemicellulose B1. The substance was soluble with difficulty in water, but its solutions were too strongly coloured to allow of a determination of optical rotation. The solution had no reducing action and gave no definite colour with iodine. It was easily hydrolysed to reducing sugars and gave a positive naphthoresorcinol test and a faint green colour with the orcinol reagent.

Determinations of furfuraldehyde and carbon dioxide in the usual manner gave: furfuraldehyde, 2.35 %, 2.32 %; carbon dioxide, 2.39 %, 2.47 %, equivalent to uronic anhydride, 9.56 %, 9.88 %. The furfuraldehyde yield was thus almost wholly accounted for by the uronic anhydride present, the balance corresponding to less than 1 % of pentosan. It was therefore concluded that pentose units were absent from this hemicellulose, which was composed of hexose and uronic anhydride units only.

A study of the rate of hydrolysis of the substance by dilute acid showed that hydrolysis was practically complete within 3 hours, using 3.5 % sulphuric acid (Table III).

Table III.

Time of hydrolysis minutes	% reducing sugar liberated, calc. as glucose (Bertrand)	
	1.4 % sulphuric acid	3.5 % sulphuric acid
30	24.2	40.6
45	45.4	—
60	50.2	59.5
90	50.2	62.7
120	49.9	67.6
160	—	79.8
190	—	84.7
220	—	90.0
250	—	86.2

Accordingly, 2 g. of the hemicellulose were hydrolysed in this manner. During the hydrolysis, a small amount of an insoluble flocculum separated, and this was found to be unhydrolysed by 5 % sulphuric acid in 5 hours. (Some evidence was obtained that the amount of this product formed was dependent upon the conditions of drying the hemicellulose. Samples dried directly in the steam-oven, whereby the hemicellulose was obtained as a horny mass, gave as much as 17 % of the insoluble material, while those dried gradually through alcohol and ether gave only 6 % or less. Anderson [1931, 1, 2], working with hemicelluloses from cottonseed hulls, obtained 19 % of a "body X," apparently an impurity similar to this insoluble product; he considered it to be an essential part of the hemicellulose.) After hydrolysis, the greater part of the acid was removed by means of barium hydroxide; the filtrate was then warmed with excess of barium carbonate, filtered and the filtrate evaporated to a syrup *in vacuo*. The syrup was extracted several times with 85 % alcohol, and in the alcoholic solution the following sugars were identified.

Mannose: as phenylhydrazone. After removal of the phenylhydrazone, no glucosazone was formed on heating with excess of phenylhydrazine; glucose was therefore absent.

Galactose: as osazone, after removal of mannose; as mucic acid, by oxidation with dilute nitric acid.

Tests for other hexoses and pentoses were entirely negative.

The uronic acid present in the hydrolysate was isolated in the form of its barium salt by a method similar to that of Dickson *et al.* [1930]. From 10 g. hemicellulose B1, hydrolysed for 4 hours with a mixture of 12 g. sulphuric acid and 500 cc. water, 1.5 g. of a barium salt were obtained. This was found to contain 24.7 % Ba (barium uronate contains 26.1 % Ba). From this, a cinchonine salt of the uronic acid was prepared by the method of Nanji [1933], and had M.P. 172°, agreeing with that of a cinchonine salt prepared similarly from an authentic sample of galacturonic acid. On hydrolysis by the hydrobromic acid-bromine method of Heidelberger and Goebel [1927], crystals of mucic acid, M.P. 216°, were obtained, thus giving sufficient evidence of the presence of galacturonic acid, since galactose itself is not oxidised to mucic acid by the reagent used.

From these results it was concluded that the hemicellulose B1 of *Cetraria islandica* was a galactose-mannose-galacturonic acid complex. An estimation of the approximate amount of each of these units present in the hydrolysis mixture was made, mannose being estimated as its phenylhydrazone, and (mannose *plus* galactose) by copper reduction, after removal of uronic acid as the barium salt; the details of the methods used were those given in Browne's *Handbook of Sugar Analysis*. The average values obtained were: anhydromannose, 39.6 %; anhydrogalactose, 44.3 % (uronic anhydride, 9.74 %).

Partial hydrolysis of hemicellulose B1. It was noted during the study of the rate of hydrolysis of the hemicellulose by acids (Table III) that with 1.4 % sulphuric acid hydrolysis to the extent of about 50 % took place within 90 minutes, but that further treatment led to no increase in reducing sugars, but rather to a slight fall, due probably to the destruction of uronic acid [*cf.* Preece, 1931]. In order to study this apparent partial hydrolysis more fully, two portions, each of 3 g. of the hemicellulose, were hydrolysed with 2 cc. sulphuric acid and 250 cc. water for 90 minutes. The hydrolysate was in each case neutralised with barium hydroxide followed by barium carbonate, the precipitated barium sulphate with excess carbonate well washed with hot water, and the clear filtrates concentrated to 50 cc. *in vacuo* at low temperature. On

pouring the syrup into two volumes of alcohol, a brownish gum was thrown down, which remained without granulating when left in alcohol at 0° overnight. Two reprecipitations from aqueous solution by alcohol, however, sufficed to granulate the material, which was then dried by means of absolute alcohol and finally *in vacuo*. The amount of the product obtained was (a) 1.05 g.; (b) 1.51 g. From the alcoholic liquors of the second batch, 1.47 g. of sugars remained on evaporation.

(a) *Sugars*. In the alcohol-soluble fraction (sugars) only galactose could be identified. No trace of mannose phenylhydrazone was given under the usual conditions. It therefore appeared that galactose was the only sugar liberated by gentle hydrolysis.

(b) *Insoluble gum*. This fraction, when dry, was found to contain 5.8 % Ba—a figure far too low for a barium uronate and indicating that a fairly complex molecule still remained. After hydrolysis of the gum with 5 % sulphuric acid for 3 hours, mannose and galactose were identified in the hydrolysis mixture, and a quantitative determination showed that they were present roughly in the ratio 3 : 1. A carbon dioxide estimation on the original gum gave 8.6 % uronic anhydride (ash-free). The question of the structure of this gum, and of the parent hemicellulose, is discussed below.

Hemicellulose B2. This fraction resembled B1 in appearance and similarly gave a dark-coloured solution in water. It again had no reducing action but readily gave reducing sugars on gentle hydrolysis. Furfuraldehyde and carbon dioxide determinations gave the following results: furfuraldehyde, 1.72 %; carbon dioxide, 1.97 %, 2.06 %, equivalent to uronic anhydride, 7.88 %, 8.24 %. Again, the furfuraldehyde yield was almost completely accounted for by the uronic anhydride present, so that pentose groups were absent from this hemicellulose also.

The results of a study of the rate of hydrolysis by acids is shown in Table IV.

Table IV.

Time of hydrolysis minutes	% reducing sugar liberated, calc. as glucose (Bertrand)	
	1.4 % sulphuric acid	3.5 % sulphuric acid
30	15.2	32.1
60	24.1	57.1
90	37.1	74.3
120	54.1	87.7
150	54.5	90.1
180	—	89.2
200	74.2	—
240	86.1	—
300	88.2	—

In the case of this substance, there was no evidence of any definite intermediate stage of hydrolysis by the weaker acid; on the whole, the substance seemed less resistant to acid hydrolysis than hemicellulose B1.

The sugars present were identified by the same methods as in the previous case, mannose and galactose being the only sugars found. The small amount of material available made impossible the isolation of the uronic acid as a cinchonine salt; however, a barium compound was obtained, which on oxidation with dilute nitric acid gave mucic acid, so that it was concluded that galacturonic acid was again present.

Estimations of the sugars present were carried out as described above, and the results are shown in Table V. There seemed to be a rather wider variation than usual between the amounts of the sugars found in two samples.

Table V.

Sample	Anhydromannose %	Anhydrogalactose %	Uronic anhydride %
1	21.1, 23.9	64.6	7.88
2	17.6, 17.6	60.1, 62.8	8.24

Hemicellulose extracted by 17 % alkali. This material, after purification by means of its copper compound, was obtained as a light-coloured powder, similar to the other hemicelluloses. It yielded 1.60 % of furfuraldehyde, and carbon dioxide corresponding to 10.7 % uronic anhydride; no pentose was therefore present. The sugars were again identified as mannose and galactose only, and the uronic acid as galacturonic acid by methods similar to those employed in the case of hemicellulose B1. Similar rough estimations of the proportions of the sugar units present gave: anhydromannose, 22.9 % anhydrogalactose, 62.9 %, figures very close to those obtained in the case of hemicellulose B2.

The course of acid hydrolysis of this substance was examined as before, the results being shown in Table VI.

Table VI.

Time of hydrolysis minutes	% reducing sugar liberated, calc. as glucose (Bertrand)	
	1.4 % sulphuric acid	3.5 % sulphuric acid
30	—	26.0
60	11.3	32.0
90	26.0	34.5
105	—	41.0
120	27.3	42.1
150	31.0	46.2
180	33.0	54.8
240	34.1	58.9
300	—	61.3

The substance was evidently much more resistant to acid hydrolysis than either of the hemicelluloses previously studied; it was apparently hydrolysed only to the extent of about one-third by the weaker acid, and again it was found that the only sugar set free during this partial hydrolysis was galactose. On further treatment of the portion unhydrolysed by 1.4 % acid within 4 hours, mannose and galactose were found in the hydrolysis-liquor in the proportion 1 : 2. In all cases examined, the uronic anhydride content of the products of partial hydrolysis was low (3–5 %), suggesting that very extensive decarboxylation had taken place under the influence of the acid, although the hexose units remained to some extent in combination.

Action of enzymes on the hemicelluloses. In view of the large amount of work recorded on the action of diastases on the water-soluble carbohydrates of *Cetraria*, a few experiments were made to test the action of these enzymes on the different hemicelluloses. Malt diastase was without action on any of the substances, but takadiastase produced about 30 % hydrolysis (measured by copper reduction) in 8 days in the case of hemicellulose B1; it was without action on the hemicellulose extracted by 17 % alkali. Attempts to isolate the products of the action of the enzyme failed.

6. *The cellulose residue.*

After very prolonged extraction with 17 % sodium hydroxide (lasting in all about 3 weeks), the residue was well washed with dilute acetic acid, until free from alkali, then with hot 50 % alcohol to remove sodium acetate and finally dried by means of alcohol, giving a dark brown fibrous material.

This substance swelled and dissolved slowly in Schweitzer's reagent and, with the exception of a very small dark residue, was soluble in zinc chloride-hydrochloric acid. On hydrolysis by the method of Monier-Williams [1921] it gave reducing sugars to the extent of 80 % (as glucose); from the hydrolysis mixture, a small amount (about 2 %) of mannose phenylhydrazone was obtained and large amounts of glucosazone, but no evidence of the presence of galactose could be found. It appeared that at least 80 % of this residue gave glucose on hydrolysis and could thus be regarded as cellulose.

A portion of the residue remained unattacked by the acid in the Monier-Williams hydrolysis, 0.4 g. of solid remaining from 5.1 g. of the residue. Similarly, on acetylation of 10 g. of the residue, 0.95 g. of substance remained insoluble in chloroform. It seemed therefore that about 10 % of the cellulose residue, *i.e.* about 0.8 % of the original dried lichen, consisted of this resistant, non-cellulosic material. It seemed possible that this substance was of lignin nature. Its dark colour precluded examination by any of the usual colour tests, and determinations of carbon and hydrogen gave figures intermediate between those required for cellulose and those accepted as an average for lignin, *viz.* C, 58.4 %; H, 6.1 %. A similar product obtained by Monier-Williams hydrolysis of a sample of lichen which had been extracted by water only (to avoid demethoxylation by alkali) was found to contain no methoxyl groups—always present in lignins. Thus the evidence was that the resistant material was not lignin. Finally, sections of the lichen failed to show the presence of lignin by the ordinary staining tests. There seemed no doubt therefore that the lichen contained no lignin, and the nature of the resistant material remained undetermined.

DISCUSSION.

1. *Structure of the hemicelluloses.*

As shown above, approximately 90 % of hemicellulose B1 was accounted for as anhydromannose, anhydrogalactose and galacturonic anhydride, the ratio of the three units being approximately 8 : 10 : 2 (see however below). The nucleus stable to hydrolysis by 1.4 % acid was found to contain anhydromannose and anhydrogalactose in the ratio 3 : 1, with 8.6 % galacturonic anhydride. This product was apparently one of the complex acid substances which have often been isolated from hemicelluloses and gums [*cf.* Norman, 1929], although more complex than the aldobionic acids recognised as the nucleus of certain of the polyuronides. During mild hydrolysis of the hemicellulose, some two-thirds of the galactose was removed, none of the mannose and approximately half the uronic anhydride. That it was impossible to detect galacturonic acid in the hydrolysis mixture was not altogether surprising, in view of the rather unstable nature of these acids and the small amounts of substance used.

Combining the above figures, one may suggest that the original hemicellulose consisted of a resistant nucleus, containing 8 or 9 anhydromannose, 3 anhydrogalactose and 1 galacturonic anhydride units, together with a less resistant portion composed of 6 or 7 units of anhydrogalactose and 1 of galacturonic anhydride. More precise statements as to the proportions of the indi-

vidual units are not warranted, on account of the relatively approximate nature of the experimental methods involved.

A similar consideration of the figures obtained in the case of hemicellulose B2 suggested a molecule based on 9 units of anhydrogalactose, 3 of anhydromannose and 1 of galacturonic anhydride. In this case, however, there was no evidence of the presence of a stable nucleus in the molecule.

The "combined" hemicellulose again was found to be based on the same three units, in approximately the same proportion as in B2. Here, however, not only was the whole molecule rather more stable towards acids, but there was evidence of a more resistant nucleus, as in B1. Since, as shown in Table VI, the apparent end-point of hydrolysis depends on the strength of acid used, there is perhaps little justification for attempting to assign to the resistant nucleus a formula based on data given by a study of the action of acid of an arbitrarily chosen concentration. All that can be said is, that the hemicellulose contains a more resistant portion containing mannose, galactose and galacturonic anhydride, and a less resistant portion consisting of galactose and galacturonic anhydride only.

Three hemicelluloses have therefore been shown to be present in the cell-walls of *Cetraria islandica*, all based upon mannose, galactose and galacturonic acid in varying proportions. The dissimilarity between fractions B1 and B2 affords some evidence for the efficiency of the method of fractionation. It may be noted that, although these products were isolated by the methods used by Norris and Preece [1930], they differed widely in their chemical nature from the products obtained by these authors, which again supports the view, often expressed, that physical nature, rather than ultimate chemical constitution, determines the behaviour of a hemicellulose.

The most notable difference between these polyuronides and those generally obtained from the cell-walls of higher plants was the complete absence from the former of any pentose units. With the exception of certain hemicelluloses of seeds, which may contain hexose or hexose *plus* uronic acid units only, it seems that practically all the hemicelluloses so far isolated from widely different plants contain pentose groups in their molecule. The hemicelluloses of seeds are perhaps of definite "reserve carbohydrate" rather than "structural" nature; it may be that these lichen hemicelluloses combine the functions both of structural and reserve carbohydrate. It is at the moment impossible to amplify this suggestion; it would, however, be strange if the lichen, with its enormous reserves of glucose in the form of lichenin, should need to call upon the mannose and galactose of its hemicelluloses.

The only uronic acid detected was galacturonic acid, and, although mannose was present in quantities comparable with those of galactose, the corresponding uronic acid was not found. This fact, together with the observation that no mannose was liberated on mild hydrolysis, led to the suggestion that the mannose residues, after entering into combination, are in some way protected from oxidation (in the plant) and from hydrolysis (in the liberated hemicelluloses). It may be noted that, although the greater part of the total carbohydrate of *Cetraria islandica* consists of glucosans (lichenin *etc.*), no glycuronic acid has been detected, unless it be that the small amount of carbon dioxide liberated from the lichenin fraction was derived from this acid. One may suggest either that the glucose molecule when present in glucosans is rather less readily oxidised than that of galactose—the apparent non-existence of a highly oxidised glucosan corresponding to pectin is interesting in this connection—or else that glycuronic anhydride complexes pass into pentosans more readily than those of

galacturonic anhydride. This second suggestion certainly does not seem to apply in the present case, since pentosans are virtually absent from *Cetraria islandica*.

2. The cell-wall of *Cetraria islandica*.

The work here described, and that previously published, has established the presence of four main well-defined groups of carbohydrate in the cell-wall of *Cetraria islandica*, viz. lichenin, isolichenin, hemicelluloses and cellulose; and the absence of pectins, pentosans and lignin. Of the carbohydrates present, lichenin, isolichenin and cellulose represent condensation products containing glucose only as the parent sugar. The hemicelluloses (using the term in the general sense, to indicate substances resistant to water-extraction, but extracted from the tissue by dilute alkali), on the other hand, are based on mannose and galactose, with partial oxidation of the latter to its uronic acid; glucose is absent. Considerable differences exist, therefore, between the cell-wall constituents of this lichen and those of a typical higher plant. These differences must be accounted for by a different metabolism governing the formation of the cell-wall. The lichens present difficulties, which do not arise in the case of the higher plants, on account of their symbiotic nature; two distinct types of cell-wall, between which considerable differences of nature might be expected to exist, are therefore present. According to Escombe [1896], Wisselingh [1898] and Wester [1909], the cell-walls of the hyphal tissues of lichens contain no cellulose but are frequently composed of chitin; no chitin has, however, been found in *Cetraria islandica*, although it is met with in other members of the genus. The hyphal membranes of this lichen are, in fact, somewhat unusual, in being composed mainly, as Escombe showed, of lichenin and isolichenin. The algal cell-walls were, on the other hand, considered by Escombe to be composed of a type of cellulose. Although it is as yet impossible to demonstrate that the hemicelluloses also occur in these gonidial walls, analogy with the higher plants suggests that such is the case. A small amount of the glucose synthesised by the algal cells becomes converted, by means at present unknown but presumably the same as those effective in the higher green plants, into galactose and mannose, thus giving the nucleus of the hemicelluloses.

In common with all northern lichens, *Cetraria islandica* has a very slow growth. The metabolic demands of the hyphae on the carbohydrate synthesised by the algal cells are thus small, with the result that the major part of the glucose undergoes condensation to lichenin and isolichenin, which are deposited on the much-thickened hyphal walls. In view also of this general low rate of metabolism, oxidation of the carbohydrates deposited in the algal cell-walls only proceeds to a very slight extent, so that the uronic anhydride content of the hemicelluloses is low.

The explanation of the absence from the plant of pentosans and pectic substances probably lies in this low oxidation. In the pectins, oxidation of the sugar units has proceeded to a very considerable extent, some 80 % of the galactose having been oxidised to uronic anhydride; such compounds could only be formed in tissues where the oxidation processes are extensive. Norman [1929] remarks that "the formation of pectin in the plant appears to take place when metabolism is at its highest peak, that is, when growth is most rapid." Further it seems that reactions leading to pectin formation take place only in tissues where there is an abundant water supply. The principal habitat of *Cetraria* is in exposed places in sub-arctic regions; having no root system, it is dependent for its water supply on absorption over its whole surface during rain and storage within its tissues of the water so absorbed. Thus it is pre-

dominantly of xerophytic habit, and another of the conditions for pectin formation is lacking.

The pentosans are generally considered to arise from condensed uronic acids by some process of decarboxylation, either by mild oxidation [Norman and Norris, 1930] or other means. Whatever the means, the process does not take place in this lichen, since pentosans are virtually absent.

3. *The relation between pectins and hemicelluloses.*

The view has frequently been expressed that some at least of the hemicelluloses may arise in the higher plants by partial decarboxylation of the pectins. This suggestion has been based on the observation that in non-lignified tissues pectins predominate over hemicelluloses, while in older, lignified tissues, the reverse is the case, rather than on any direct observations of a change in the amounts of the two classes in the same tissue at different stages of growth. Other support for the view has been found in the production from pectin, *in vitro*, of substances resembling hemicelluloses in having a low uronic anhydride content [Candlin and Schryver, 1928; Norman and Norris, 1930]. So far as *Cetraria islandica* is concerned, however, the facts given in this communication do not support this view. Hemicelluloses containing galacturonic acid occur in fair quantity in the lichen, and if there had been any direct connection between these substances and pectic bodies, as regards their origin, then the large quantities of lichen employed, containing plant specimens of all ages, would have been expected to yield a small quantity of pectin at least; such was not the case. In addition, as no lignin was detected, any pectin present in the young tissues could not have been converted into this substance as has sometimes been suggested.

It might be argued that the absence both of pectins on the one hand, and pentosans and lignin on the other, could be regarded as evidence of a connection between these classes. This may indeed be the case. But the facts presented above make it necessary to emphasise the fact that not all polyuronides, of low uronic anhydride content, arise from pectins by decarboxylation. The only conclusion, then, is that the hemicelluloses of this lichen do not arise from pectic substances, but by feeble oxidation of condensed hexoses—a process distinct from, though largely parallel with, that which leads to the formation of pectins in plants whose general level of metabolism is higher.

SUMMARY.

1. The cell-wall constituents of *Cetraria islandica*, other than lichenin and isolichenin, have been shown to consist mainly of hemicelluloses and cellulose; pectins, pentosans and lignin are absent.
2. Three distinct hemicelluloses have been isolated, one of which is more closely associated with the cellulose and resists extraction with 4 % alkali.
3. All three hemicelluloses give, on hydrolysis, mannose, galactose and galacturonic acid; the amount of the last is in each case low (10 %).
4. Evidence is given to show that in two of the hemicelluloses, the molecule consists of a resistant acid nucleus, consisting of mannose, galactose and galacturonic acid units, and a more loosely combined portion, containing no mannose.
5. The relation between the chemical nature of the cell-wall constituents and the habit of growth of the lichen is discussed.

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CCXXXI. THE INFLUENCE OF VITAMIN C ON INTRACELLULAR ENZYME ACTION.

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(Received August 22nd, 1933.)

THE discovery of glutathione by Hopkins [1921], and the recognition that it is the natural activator of certain intracellular proteolytic enzymes in animals as well as in plants [Waldschmidt-Leitz *et al.*, 1930; Grassmann *et al.* 1930; 1931], has established a relationship between these enzymes and the oxidation-reduction processes in the cell. In the ascorbic acid (vitamin C), isolated and crystallised from suprarenal gland, oranges and cabbage, Szent-Györgyi [1928; 1932; 1933] has recently found another constituent of the cell which assists in determining the oxidation-reduction potential and which may be reversibly oxidised or reduced.

Ascorbic acid in the organism is not limited solely to antiscorbutic action but has a still more comprehensive rôle. By its reversible oxidation and reduction, it is indirectly related to cell respiration and is also a determining factor in establishing the equilibrium between SH and SS compounds. The vitamin assumes a protective rôle against the oxidation of SH compounds in the organism¹. The physiological significance of these two substances (glutathione and ascorbic acid) as catalysts of oxidation-reduction processes has been considerably increased through the discovery that the activity of certain intracellular enzymes is dependent on the presence of definite oxidation-reduction potentials.

Arginase is one of the most important enzymes of intermediary protein metabolism, whose activity, according to Waldschmidt-Leitz *et al.* [1933] and Edlbacher *et al.* [1925; 1927; 1932; 1933] is dependent upon oxidation-reduction potentials. This enzyme is also activated by the system, ascorbic acid *plus* iron as is shown by the following experiment.

Example. 0.25 cc. glycerol-liver-suspension (1:10), 4.5 cc. water, 10 cc. 1 % arginine carbonate, 5 cc. 0.1 *N* glycine buffer (p_H 9.5), incubated 60 minutes at 30°; addition, 20 mg. cysteine-HCl, previously neutralised, or 20 mg. crystalline ascorbic acid (prepared from oranges), or 0.5 cc. 0.1 *N* FeSO₄; incubated 60 minutes, p_H 7.

Addition	Arginase activity (cc. 0.02 <i>N</i> NH ₃)
None	7.0
Iron	8.0
Cysteine-iron	15.0
Ascorbic acid (crystal pulp)	12.0
Ascorbic acid-iron	16.0

¹ This may explain the high concentration of sulphydryl compounds in the suprarenal gland.

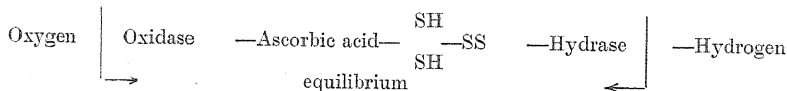
It is known that intracellular proteolytic enzymes of the cathepsin type require the presence of sulphydryl compounds for their activation. Ascorbic acid, by virtue of its oxidation-reduction potential, also apparently regulates the relation of SH to SS as is shown by these experiments. The decomposition of proteins is thus dependent on the presence of vitamin C. The conception, therefore, of a regulation of the intracellular protein metabolism by the interaction of these two substances, glutathione and ascorbic acid, in the sense of hydrolysis on the one hand and of synthesis on the other, possesses a physiological significance. It remains to be determined [cf. Grassmann *et al.* 1931] whether sulphydryl compounds alone are responsible for the activation of the catheptic enzyme systems.

Example. 5 cc. carcinoma-glycerol suspension (1:10), 0.40 g. gelatin, incubated 24 hours at 30°, p_H 4.0, total volume 25 cc. Results given are for 10 cc. of the mixture.

Additions	NH ₂ increase (cc. 0.05N KOH)
None	0.75
Cysteine (20 mg.)	1.70
Ascorbic acid (20 mg. crystal pulp)	1.60

There is scarcely a doubt that, in addition to the catheptic enzymes and arginase, other intracellular enzymes require the participation of these two substances for their activation. Among the enzyme groups which we are investigating from this point of view in our laboratories, methylglyoxalase is of particular interest. If it should turn out that the action of this enzyme is related to the oxidation-reduction potential in the cell, a new explanation would be found for the excess glycolysis in anaerobically developing cells, *e.g.* in malignant tumours.

These findings, by which for the first time a relationship is established between a vitamin and the intracellular enzymes of metabolism, are of particular significance for the question of the cause of autolysis in dying cells, which plays such an important part in malignant tumours. The much disputed question of the formation and occurrence of sulphydryl compounds in living and dying cells is perhaps plausibly explained by the following working hypothesis for respiration.



The solution of this question in connection with the activation relationships of intracellular enzyme systems, particularly those of the carbohydrate-degrading enzymes in healthy muscle and malignant tumours, will be the object of further experiments.

I am greatly indebted to Dr Ellice McDonald for his advice and assistance.

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CCXXXII. PHENYL ISOCYANATE PROTEIN COMPOUNDS AND THEIR IMMUNOLOGICAL PROPERTIES.

II. THE GELATIN COMPOUNDS.

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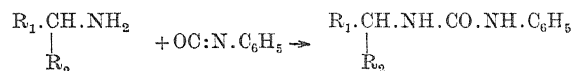
(Received August 31st, 1933.)

NATIVE proteins, with a few exceptions, when injected into the animal body readily produce antibodies. By a comparison of the chemical constitution and properties of various proteins it is possible to say that the presence of a certain amino-acid or grouping is not essential for antigenic power, and, in some instances, to offer a feasible explanation for the non-antigenicity of a certain protein. Thus, although zein is deficient in tryptophan and lysine, and caseinogen in cystine, these two proteins are markedly antigenic, and the conclusion can be reached, therefore, that the three amino-acids mentioned are not essential for antigenicity. The protamines [Wells, 1913; *cf.* review by Wells, 1929], gelatin [Wells, 1908; 1916; Landsteiner, 1917; Starin, 1918; Kahn and McNeil, 1918] and the closely related preparation, glue [Ramsdell and Walzer, 1927], on the other hand, are non-antigenic. Gelatin appears to be of special interest in this respect, and the non-antigenicity of this protein has attracted much attention. Starin [1918], in particular, carried out an extensive investigation, using the precipitin, anaphylactic, complement fixation and meiotagmin reactions, and decided that the injection of gelatin into rabbits, guinea-pigs and dogs failed to produce antibodies to gelatin. This failure of gelatin to incite antibody production has been interpreted in several ways, but the view most commonly held suggests that the non-antigenicity in this instance is due to the absence of aromatic groupings, for gelatin is deficient in tyrosine and tryptophan, and it contains only a very small amount of phenylalanine. Gelatin, however, differs from other proteins in many respects, and several additional suggestions might be advanced to account for its peculiar immunological properties. Thus, it is important to know whether the failure to produce antibodies is entirely due to the absence of aromatic groupings, and for this purpose it was decided to introduce into the gelatin molecule groupings of this nature.

Landsteiner [1919] found that gelatin coupled with diazotised metanilic acid gave precipitin reactions with antisera produced by the injection of horse-serum-proteins coupled with *m*-diazobenzenesulphonic acid, and that in higher concentrations the gelatin compound specifically inhibited the precipitin reaction between these azoproteins and their antisera. Adant [1930] and Bruynoghe and Vassiliadis [1930] studied the serological properties of gelatin coupled with diazotised aniline and obtained some antibody formation when this preparation was injected. The results of these authors will be discussed more fully later on in this paper. Medveczky and Uhrovits [1931], working with benzoylated proteins, have shown that immune sera produced by the injection of the benzoylated

proteins of horse serum or of benzoylated typhoid bacilli will give good precipitin reactions with benzoylated gelatin, and that the injection of benzoylated gelatin produces anaphylaxis in animals sensitised with benzoylated typhoid bacilli. Hooker and Boyd [1933] have very recently studied the immunological properties of gelatin coupled with diazotised arsanilic acid, and have found that the injection of this preparation produces sera which give precipitin and complement fixation reactions with other proteins coupled with diazotised arsanilic acid but not with the homologous antigen (gelatin-diazo-arsanilic acid). The evidence so far available appears to show, therefore, that gelatin coupled with these new groupings can take part in precipitin and similar reactions, but evidence of the acquirement of true antigenic function does not appear to be very satisfactory.

In a previous paper [Hopkins and Wormall, 1933] the immunological and chemical properties of phenylureidoproteins have been studied. These compounds are prepared by the action of phenyl isocyanates on various proteins, a reaction used by Raper [1907] for the preparation of phenylureidopeptide compounds. By this reaction, a new grouping, $C_6H_5NH.CO-$ or a related group, is introduced into the protein molecule, and in the earlier paper evidence has been presented which strongly suggests that the introduction occurs mainly, if not entirely, at the free amino-groups of the lysine molecules. The reaction can be represented in the following manner:



In this way, aromatic groupings are introduced into the protein, and the mode of linkage is not very different from the ordinary peptide linkage which appears to link up most of the amino-acids in the protein molecule. This reaction thus offers an alternative method for studying the effect of the introduction of new groups into the protein molecule, and for several reasons it is most suitable for this purpose. The mode of linkage is not unlike that already present and the protein is not subjected to very drastic treatment. Furthermore, it appears very desirable that different methods of introducing the same new group into different parts of the same protein molecule might be studied. Experiments along these lines are being carried out and will be reported later. In the investigation described here, a study has been made of the chemical and immunological properties of phenylureido- and *p*-bromophenylureido-gelatin, the primary object being to determine whether the introduction of aromatic groupings into the gelatin molecule would render it fully antigenic. For this purpose these gelatin compounds have been tested, in precipitin and complement fixation tests, with several antisera against phenylureido-horse-serum-globulin, and immunisation experiments have been carried out to determine whether antibodies are produced when these phenylureidogelatin compounds are injected into rabbits.

EXPERIMENTAL.

Preparation of phenylureidogelatin.

4 g. of gelatin (Coignet's "Gold Label") were dissolved in 400 cc. of warm water, 200 cc. of a phosphate buffer of p_H 8.0 added, the mixture cooled and stirred and 3.2 cc. of phenyl isocyanate added. The mixture was kept at p_H 8 and stirred for about 1 hour. The phenylureidogelatin was then precipitated by acetic acid and purified by two further precipitations as described in a previous

paper [Hopkins and Wormall, 1933]. This amount of gelatin usually furnished under these conditions about 60–80 cc. of a solution containing about 2 % of protein. NaCl was added to give 0.9 %, and the solution was filtered through a Berkefeld filter-candle. The filtered solution was kept in sterile tubes in the ice-chest, and the solidified mass was melted by gentle heat when required.

The solutions thus obtained were perfectly clear and closely resembled solutions of gelatin. The phenylureidogelatin compounds, like the similar compounds of serum-globulin and caseinogen, can be precipitated by the addition of dilute acetic acid to p_H 4–4.5, and the precipitates are readily soluble again at p_H 7.5, although with the gelatin preparations dissolution is best effected by warm water or NaCl solution (25–30°). The solutions used in this work (containing 1–2.5 % of phenylureidogelatin) solidified on cooling, and thus substitution in the free amino-groups of the gelatin molecule does not destroy the capacity to form gels. In this connection, it is interesting to note that gelatin treated with nitrous acid or with formaldehyde still exhibits gel-formation.

Preparation of p-bromophenylureidogelatin.

This compound was prepared by the addition of a filtered ethereal solution of *p*-bromophenyl isocyanate (4 g. in about 30 cc. of ether) to a cooled and stirred mixture of 400 cc. of 1 % gelatin (Coignet's "Gold Label") and 200 cc. of phosphate buffer of p_H 8.0. The mixture was cooled, kept at p_H 8 and stirred for about 1½ hours, after which precipitation and purification were effected as described previously [Hopkins and Wormall, 1933]. From this amount of gelatin, 50–60 cc. of a solution containing about 2.5 % of *p*-bromophenylureidogelatin were usually obtained. NaCl was added to the extent of 0.9 % and the solution filtered through a Berkefeld filter-candle and kept in the ice-chest.

Nitrogen determinations.

The ratios of free amino-N to total N in the above preparations and in gelatin were determined, and in this way a measure of the amount of substitution was obtained, as described in the previous paper. The main bulk of the antigen solutions used for the immunisation experiments gave the following figures for the free amino-N (Sørensen's formaldehyde method) calculated as a percentage of the total N: gelatin, 3.0 %; phenylureidogelatin, 0.8 % and *p*-bromophenylureidogelatin 1.2 %. These figures indicate substitution to the extent of 70–75 % in the phenylureido-compound and about 60 % in the *p*-bromophenylureidogelatin. The last-named compound was found to contain 2.2 % of bromine, whilst another preparation of the same substance had a bromine content of 2.6 %.

Immunisation.

Three groups, each of 4 rabbits, were used for the injection of the following substances:

- Group (a) Gelatin (Coignet's "Gold Label").
- Group (b) Phenylureidogelatin.
- Group (c) *p*-Bromophenylureidogelatin.

The control injections with untreated gelatin were made in order to establish the non-antigenicity of the sample of gelatin used in this investigation. The two phenylureidogelatin compounds were prepared as described above. It was thought desirable in these experiments, where the introduction of aromatic

groupings was being studied, to avoid the use of phenol as antiseptic, and therefore all the solutions used for injection were sterilised by filtration through Berkefeld filter-candles.

At each injection the rabbits received a volume of the antigen solution containing 0.25 g. of the protein, and the injections were made at intervals of 7 or 8 days. These injections were usually made intraperitoneally, but with 50 % of the animals the last two injections were intravenous. Samples of serum from each rabbit were tested 7 or 8 days after the third, fourth, fifth, sixth and seventh injections and also about 3 weeks after the seventh injection. Precipitin tests were carried out on each occasion, and usually complement fixation tests were also made. Each serum was tested against all three antigens (gelatin, phenylureidogelatin and *p*-bromophenylureidogelatin) and against phenylureido-chicken-serum-proteins and phenylureido-rabbit-serum-proteins.

Precipitin tests. The technique of these tests was as described previously [Johnson and Wormald, 1930; Hopkins and Wormald, 1933]. Some of the tests were made at 37°, but the majority were done at room temperature (15–20°), since it was found early in this work that the gelatin antigens gave much better precipitin reactions at the lower temperatures. In many instances tests were made at 37° as well as at room temperature. The results were recorded as follows: (no reaction), f.tr. (faint trace), tr. (trace), \pm , +, $\pm\pm$, $\pm\pm\pm$ (increasing degrees of precipitation).

Complement fixation tests. These were carried out as described previously [Johnson and Wormald, 1930], the results being recorded as follows: 4 (complete haemolysis), – (no haemolysis), 3, 2 and 1 (intermediate degrees of haemolysis).

(1) *The phenylureidogelatin compounds and their reactions with antiserum to phenylureido-horse-serum-globulin.*

The preliminary observation that phenylureidogelatin preparations give precipitates with antisera to phenylureido-horse-serum-globulin, noted in a previous paper [Hopkins and Wormald, 1933], was amplified by making tests with various antisera and using much wider ranges of antigen dilution. These precipitin reactions were also compared with those obtained in similar series of phenylureido-horse-serum-proteins and phenylureido-chicken-serum-proteins tested against the same antisera. Precipitin tests were made both at 37° and at room temperature, and from the results obtained (*cf.* Table I) the conclusion was reached that room temperature is preferable to 37° for precipitin tests with the gelatin antigens. Indeed it has frequently been observed that well-marked precipitates of phenylureidogelatin *plus* antisera to phenylureido-horse-serum-globulin have become much less bulky after incubation at 37°. This was well illustrated in the experiment quoted in Table I, for on the addition of the antiserum to phenylureidogelatin good precipitates were obtained, and these became much less pronounced after 1 hour at 37° and still less marked after 3 hours at this temperature. When these antigen-antibody mixtures at 37° were cooled, the gelatin antigen precipitates became stronger again and were comparable with those at room temperature. Thus the precipitates of “phenylureido”-antiserum with phenylureidogelatin appear to be more soluble at 37°, possibly owing to the retention by the gelatin of some of its solubility properties. The other antigens used in these tests, phenylureido-chicken-serum-proteins and phenylureido-horse-serum-proteins behaved in the normal manner and with antisera to phenylureido-proteins gave precipitates at 37° which were equal to, or greater than, those obtained at room temperatures.

Table I. *Comparison of the precipitin reactions with various phenylureido-protein compounds and the influence of temperature on these reactions.*

Antigen	Antigen dilution	Immune serum (anti-phenylureido-horse-serum-globulin)					
		No. 50		No. 51		No. 53	
		16°	37°	16°	37°	16°	37°
Phenylureidogelatin	1:20	—	—	—	—	—	—
	1:100	f.tr.	—	tr.	f.tr.	tr.	—
	1:500	±	tr.	+	±	+	±
	1:2500	+	±	+	±	+	±
	1:12,500	tr.	tr.	tr.	tr.	tr.	tr.
Phenylureido-chicken-serum-proteins	1:20	±	±	+	+	±	+
	1:100	+	+	+	+	+	+
	1:500	+	+	+	+ ±	±	±
	1:2500	tr.	tr.	±	±	tr.	tr.
	1:12,500	—	—	—	—	—	—
Phenylureido-horse-serum-proteins	1:20	++	++	++ ±	++ ±	+ ±	+ ±
	1:100	++ ±	++ ±	++ ±	++ ±	++	++
	1:500	+ ±	+ ±	+ ±	+ ±	+	+ ±
	1:2500	±	±	±	±	tr.	±
	1:12,500	—	—	—	f.tr.	—	—

The results given in Table I indicate a very marked capacity on the part of phenylureidogelatin to give precipitates with antisera to phenylureido-horse-serum-globulin, but maximum precipitation is observed with much greater dilutions of the gelatin preparations compared with the similar preparations of horse-serum-proteins or chicken-serum-proteins. These differences in the zone of maximum precipitation may be related to the number of reactive groupings in the antigen, but this does not appear probable since the preparations used for these tests (Table I) did not differ to any great extent in the number of phenylureido groupings introduced. Complement fixation tests have also demonstrated the same difference in capacity to react with the antiserum, the phenylureidogelatin preparations being active in much higher dilutions than are the corresponding serum-protein compounds (*cf.* Table II).

Table II. *Complement fixation tests with phenylureidogelatin compounds.*

(Tests with immune serum, No. 51—anti-phenylureido-horse-serum-globulin.)

Antigen	Antigen dilution								Con- trol (NaCl)	Read- ing after (hr.)
	1:20	1:60	1:180	1:540	1:1620	1:4860	1:14,580	1:43,740		
Phenylureido-horse-serum-proteins	4	3	—	—	—	—	1	4	4	$\frac{1}{2}$
	4	3	1	—	—	—	2	4	4	1
Phenylureido-gelatin	4	2	—	—	—	—	—	—	4	$\frac{1}{2}$
	4	4	1	—	—	—	—	—	4	1

The other gelatin preparation used in this investigation, *p*-bromophenylureidogelatin, gave very similar results with all the antisera, but the precipitin and complement-fixation reactions with this preparation were not quite so pronounced as those with phenylureidogelatin.

Inhibition tests, involving the addition of simple substances to mixtures of the antigen and antibody in order to determine which groupings could specifically inhibit the formation of the precipitate [Landsteiner, 1920; Landsteiner and van der Scheer, 1931; 1932] were made with certain amino-acids and their

phenylureido-compounds in a previous paper [Hopkins and Wormall, 1933]. These experiments, made with phenylureido-chicken-serum-proteins and antisera to phenylureido-horse-serum-globulin, furnished very strong evidence for the view that the immunologically active grouping in the phenylureidoprotein is the phenylureidolysine group. Similar results have been obtained from tests carried out with phenylureido- and *p*-bromophenylureido-gelatin as antigens in the precipitin reaction. Experiments have also been made to determine whether similar inhibition might be obtained in complement fixation tests with the phenylureidogelatin compounds, and the results of an experiment of this nature are given in Table III. From these results it will be seen that phenylureidolysine, and to a lesser extent phenylureidoalanine, inhibit these complement fixation reactions.

Table III. *Inhibition of complement fixation tests.*

Antiserum. No. 51 (anti-phenylureido-horse-serum-globulin).

Antigen. Phenylureidogelatin.

Antigen dilution	Inhibiting substance									
	NaCl		Lysine		Alanine		Phenylureido-lysine		Phenylureido-alanine	
1:20	4	4	4	4	4	4	4	4	4	4
1:60	4	4	4	4	4	4	4	4	4	4
1:180	2	3	3	4	2	3	4	4	4	4
1:540	—	—	—	2	—	—	4	4	3	4
1:1620	—	—	—	—	—	—	4	4	1	2
1:4860	—	—	—	—	—	—	4	4	—	1
1:14,580	—	—	—	—	—	—	4	4	—	2
1:43,740	—	—	—	—	—	—	4	4	2	3
1:131,220	1	3	1	2	1	3	4	4	4	4
1:393,660	3	4	2	3	3	4	4	4	4	4
Control (NaCl)	4	4	4	4	4	4	4	4	4	4
Reading (hr.) after	$\frac{1}{2}$	1	$\frac{1}{2}$	1	$\frac{1}{2}$	1	$\frac{1}{2}$	1	$\frac{1}{2}$	1

Details of tests. A mixture of 0.5 cc. of the antigen, 0.10 cc. of the inactivated antiserum, 0.10 cc. of 1:5 guinea-pig serum and 0.10 cc. of a neutral *M*/100 solution of the "inhibiting" substance (in 0.9% NaCl solution) was kept at room temperature for 1 hour. 0.5 cc. of a 4% suspension of sensitised ox red cells was then added to each tube and the tubes placed in a water-bath at 37°.

(2) *Immunisation experiments with phenylureido- and p-bromophenylureido-gelatin.*

In order to determine whether the introduction of aromatic groupings into gelatin really endows this protein with antigenic power, immunisation experiments have been carried out with phenylureidogelatin and *p*-bromophenylureidogelatin. In these experiments the modified gelatins were injected into rabbits as described earlier in this paper, and the sera of these rabbits were tested frequently by precipitin and complement fixation tests, for the presence of antibodies to gelatin itself, to phenylureidogelatin or to any other phenylureidoprotein (phenylureido-chicken-serum-proteins, *etc.*). Each rabbit received seven injections of the phenylureido- or *p*-bromophenylureido-gelatin, at intervals of 7 or 8 days, and the sera were tested 7 or 8 days after the third and subsequent injections. Throughout the whole of each series of injections the serological tests gave negative results, although there were one or two occasions when the precipitin tests made with these sera and phenylureido-chicken-serum-proteins (or phenylureido-rabbit-serum-proteins) showed very faint traces of a precipitate. These turbidities were very slight, however, certainly not more

significant than a faint trace, and they were only observed in very strong antigen solutions (a 1 : 20 solution of the 5 % protein solution). Since even these slight reactions were never obtained with the concentrations of antigen normally used for the testing of antisera, and since they were obtained on a few occasions only, the conclusion was reached that they are not significant. The complement fixation tests made simultaneously with the same sera and the same antigen solutions were completely negative on every occasion.

DISCUSSION.

The action of phenyl *isocyanate* and *p*-bromophenyl *isocyanate* on gelatin results in the production of phenylureidogelatin compounds which readily give precipitin and complement fixation reactions with antisera to phenylureido-horse-serum-globulin. In the complement fixation tests these gelatin compounds appear to behave very much like the corresponding serum-protein compounds, but in the precipitin reactions they show certain significant differences. The chief difference is related to the influence of temperature on the extent of precipitation, the amount of precipitate formed with the gelatin compound being much less at 37° than at 16–20°. This difference is possibly related to the greater solubility of gelatin at the higher temperature. A comparison of the precipitin and complement fixation tests with phenylureidogelatin and the corresponding serum-protein compounds has also shown that the zone of maximum precipitation, or the zone of complete complement fixation, is found in much more dilute solution with the gelatin compounds. This difference in activity does not appear to be related to the number of phenylureidolysine groupings in the phenylureido-protein, and at the present time no explanation can be advanced to account for the greater power of the gelatin compounds to give precipitates in very dilute solution.

This ability to give precipitates or complement fixation when mixed with various antisera does not in any way prove that the phenylureidogelatin preparations are truly antigenic. A similar power to give precipitation with suitable antisera is exhibited by the soluble specific substances, which do not normally incite antibody response when injected into an animal [Heidelberger and Avery, 1923, *cf.* Review by Heidelberger, 1927]. More recent work suggests that some modification of the view that these immunologically active carbohydrates and similar haptens are non-antigenic, will perhaps be necessary, since it has been shown that certain of these haptens can produce antibodies when injected after adsorption on kaolin, collodion particles, charcoal, *etc.* [Gonzalez and Armangue, 1931; Zozaya, 1931; 1932; Landsteiner and Jacobs, 1932; 1933]. Any modification of this view does not affect, however, the argument advanced here that the capacity to give a precipitate or to give complement fixation when certain antisera are added does not necessarily imply that the substance is antigenic, *i.e.* that it will produce antibodies when injected into an animal. There are several other instances of precipitin reactions with substances which are not truly antigenic. Thus, antibodies to azoproteins will give precipitates with azo-dyes [Landsteiner and van der Scheer, 1932], indicating the specific combination of antibodies with substances of relatively small molecular weight to give a precipitate. The demonstration of true antigenic power involves the formation of antibodies when the substance is injected into an animal, these antibodies being detected by the precipitin, complement fixation or some similar test.

The immunisation experiments with phenylureido- and *p*-bromophenylureido-gelatin compounds described in this paper have shown that these com-

pounds do not produce any significant antibody response detectable by precipitin or complement fixation reactions. From these results the conclusion can be reached, therefore, that to render gelatin antigenic in the full sense it is not sufficient to introduce aromatic groupings. The non-antigenicity of gelatin, although it might conceivably be due in part to the absence of such groupings, is not due solely to this deficiency. For several reasons it would be desirable to introduce into the gelatin molecule an aromatic grouping which is completely or almost completely identical with one of those present in other proteins, *e.g.* the tyrosine or tryptophan groupings linked to the rest of the molecule by means of a peptide linkage. It is hoped that experiments along these lines will be made in the near future, but it is realised that it may be extremely difficult to effect the introduction of a grouping of this kind without recourse to a fairly drastic method which will involve some other change in the protein molecule.

The results obtained by Adant [1930] and Bruynoghe and Vassiliadis [1930] are difficult to correlate with those recorded in this paper. Adant found that gelatin coupled with diazotised aniline produced, when injected into the rabbit, antibodies which would give precipitates with gelatin-diazotised aniline and also with gelatin itself. The reaction between these antisera and gelatin is most difficult to explain, and it appears necessary to postulate the acquirement of antigenic power when diazotised aniline is coupled with gelatin and the formation of at least two types of antibody or antibody groupings when this complex is injected; one antibody which can specifically react with any diazotised aniline-protein compound and another which can specifically react with gelatin. Bruynoghe and Vassiliadis [1930] carried out complement fixation tests with these antisera to gelatin-diazotised aniline and obtained fixation with gelatin and not with gelatin-diazotised aniline. These findings also are difficult to interpret and without further experimental details it would be impossible to assess the significance of these complement-fixation results. Hooker and Boyd [1933] have recently carried out similar investigations using gelatin-diazotised arsanilic acid, and they find that this gelatin compound produces in the rabbit antibodies which will give precipitin reactions with other proteins coupled with diazotised arsanilic acid but not with gelatin-diazotised arsanilic acid or with gelatin. These results of Hooker and Boyd indicate that antigenicity is to some extent conferred on gelatin by coupling it with diazotised arsanilic acid, but, as these authors point out, the results "do not show that the non-antigenicity of gelatin is due solely to its deficiency in aromatic amino-acids." Hooker and Boyd record the significant finding, but one which is difficult to interpret, that gelatin-diazotised arsanilic acid reacts with antisera to egg-white-diazotised arsanilic acid but not with antisera to gelatin-diazotised arsanilic acid, although the last-named antisera give precipitates with egg-white proteins (or caseinogen) coupled with diazotised arsanilic acid. From many points of view, it might be claimed that the method used in the work described here has several advantages over other methods which have been used for the introduction of aromatic groupings into the gelatin molecule. With the phenyl *isocyanate* method, the linkage is one which does not differ very considerably from the ordinary peptide linkage, and a second point in its favour is that any excess of phenyl *isocyanate* is rapidly destroyed and the products formed from it are easily removed from the phenylureidogelatin solution.

From the immunisation experiments described here, it appears probable that some explanation other than that relating to aromatic groupings will have to be advanced to account for the non-antigenicity of gelatin, but any suggestion offered at the present time will be mainly speculative. The chemical constitutions

of gelatin and other proteins can be compared and the non-antigenicity of the former attributed to the absence of some substance or grouping which is present in all antigenic proteins. Gelatin is deficient in, amongst other things, aromatic amino-acids and carbohydrate groupings. The mere introduction of benzene rings does not appear to convert gelatin into an antigen, and with reference to the second possibility there appears to be little or no evidence that proteins must contain a carbohydrate grouping before they are antigenic. The carbohydrates associated with certain bacterial proteins undoubtedly play the predominant rôle in the determination of certain types of specificity, and there are probably very many instances of specificity determined by the carbohydrates present. It does not necessarily follow, however, that protein specificity is always determined by the carbohydrate groupings which are usually associated with native proteins, nor does it mean that antigenicity is exhibited only by those proteins which contain carbohydrate groupings. In this case, however, as with the other suggestions which might be offered to account for the non-antigenicity of gelatin, it does not appear profitable at the present time to discuss the matter at any length, and much additional evidence will be needed before it is possible to reach any final decision.

SUMMARY.

1. Phenylureido- and *p*-bromophenylureido-gelatin have been prepared by the method previously described. Some properties of these compounds are described.
2. These gelatin preparations give marked precipitin and complement fixation reactions with antisera to phenylureido-horse-serum-globulin.
3. With the gelatin preparations the zone of maximum precipitation is found in higher dilutions than are the corresponding zones for phenylureido-compounds of horse-serum-globulin or of caseinogen. This difference does not appear to be related to the number of new groupings introduced.
4. The precipitin reactions with these gelatin compounds are most pronounced when carried out at room temperature (15–20°) or at lower temperatures, since there is a strong tendency for the precipitates to go into solution at 37°.
5. Phenylureido- and *p*-bromophenylureido-gelatin when injected into rabbits over a long period fail to produce antibodies which are detectable by precipitin and complement fixation tests. The conclusion is reached, therefore, that the non-antigenicity of gelatin is not due solely to a deficiency in aromatic groupings.

One of the authors (A. W.) is indebted to the Medical Research Council for a part-time personal grant and for a grant which has, in part, defrayed the expenses of this research.

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CCXXXIII. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

XII. THE PREPARATION AND METABOLISM OF *d*-ACETYLCYSTEINE.

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(Received September 1st, 1933.)

THE racemising action of acetic anhydride on amino-acids has attracted a considerable amount of attention recently. For this reason and also because du Vigneaud and Sealock [1932] have suggested that the acetylcysteine made by one of us [Pirie, 1931, 1] was partly racemic it seemed advisable to study this substance further. The metabolic work which we carried out with this doubtful material [Hele and Pirie, 1931] had also to be repeated.

Using the method of preparation already described, very little racemisation does in fact occur; the preparation has, however, been simplified by the substitution of lead acetate for cuprous oxide as a precipitant for the acetylcysteine. The use of ketene as an acetylating agent was suggested to us by the work of Bergmann and Stern [1930]. The reaction proceeds very smoothly in alkaline solution and gives, as is shown later, an unracemised product in nearly quantitative yield.

The fact that the cysteine in mercapturic acid is acetylated gives a special interest to metabolic studies on acetylcysteine. The results of previous papers on the metabolism of related compounds [Hele and Pirie, 1931; Pirie, 1932] may be summarised as follows:

Substance	% excreted as sulphate in 2 days	% excreted as neutral sulphur in 2 days
Cysteine or cystine	70	4
Glutathione	72	10
Glycylcysteine	56.5	19.5
Methionine	66	16

The present work on acetylcysteine gives the following mean figures. After oral administration 48 % of the dose is excreted as sulphate and 32 % as neutral sulphur; after subcutaneous administration the figures are 28 and 42 %. Our previous results with orally administered acetylcysteine were similar but on the single occasion when it was given subcutaneously a larger percentage was oxidised. It is clear that acetylation has reduced considerably the oxidisability of the molecule.

Preparation of acetylcysteine. A brisk current of ketene, prepared by the method of Ott *et al.* [1931], is passed into a cold suspension of 20 g. of cystine in 130 cc. of 0.6 N NaOH. The mixture is kept cool with tap water and after

half an hour 30 cc. of 2.5 *N* NaOH are added. All the cystine soon goes into solution and after about an hour the action is complete, *i.e.* a sample has no formaldehyde titration. The solution is now slightly acid, 20 cc. of glacial acetic acid and some zinc are added and the mixture warmed to 50–60° and allowed to cool. It is stirred occasionally and filtered when cold.

The filtrate is made up to about 400 cc., and normal lead acetate solution is added till the supernatant fluid after centrifuging a sample gives no further precipitate with more lead acetate. The precipitate is centrifuged and washed two or three times on the centrifuge with 1 % lead acetate solution; it is then suspended in water and decomposed with hydrogen sulphide. There should be no avoidable delay during the operations described in this paragraph or a brown colour will develop, and the yield will be small.

The lead sulphide is removed by filtration and the filtrate distilled to small bulk *in vacuo*. Acetylcysteine crystallises readily in thick prisms and occasionally in well-formed octahedra; yield 21.5 g.

The product made in this way is purer than that obtained by the old method; its m.p. is 111° instead of 107–109°, and a 2.7 % solution in water has $[\alpha]_{546}^{20} = +6.3^\circ$.

1.28 g. of this *d*-acetylcysteine were hydrolysed by boiling for 2 hours with 20 cc. of 6 *N* HCl and evaporated to dryness. The residue was taken up in water, neutralised and aerated after adding a trace of iron. 0.95 g. of cystine was obtained; this is 94 % of the theoretical amount. A 0.4 % solution in *N*/9 HCl showed $[\alpha]_{546}^{20} = -274^\circ$. This value is that to be expected for a dilute solution of cystine in weak acid [Pirie, 1931, 2]; it is clear therefore that, when prepared by the ketene method, acetylcysteine undergoes little or no racemisation.

If a ketene lamp is not available an almost equally unracemised product may be made, though rather less conveniently, with acetic anhydride as in Nicolet's [1930], Hollander and du Vigneaud's [1931] and Pirie's [1931, 1] methods if the reaction mixture is kept cold. The reduction and precipitation with lead are carried out exactly as in the method described in this paper. A sample of acetylcysteine prepared in this way was hydrolysed with 6 *N* HCl: the cystine obtained from it had $[\alpha]_{546}^{20} = -265^\circ$. Under these conditions therefore racemisation, as might be expected from the work of Bergmann and Zervas [1928], does not occur very readily.

Diacetylcysteine is easily prepared from acetylcysteine by oxidation with hydrogen peroxide. Experiments carried out in the same manner as those already published by one of us [Pirie, 1931, 2; 1933] show that this oxidation is catalysed by iron and thiocarbamide in much the same way as the oxidation of glutathione, but that copper has only a slight catalytic power. The oxidation is therefore carried out in the same way as that of glutathione [Pirie, 1931, 1] but using a trace of iron as catalyst. Diacetylcysteine prepared in this way, like that prepared by Hollander and du Vigneaud [1931], is a non-crystalline, slightly gummy solid, exceedingly soluble in water.

Metabolic experiments.

Two bitches, Patsy (8.5 kg.) and Christina (8.7 kg.), were used. The diets and analytical methods were the same as in our earlier work [Hele and Pirie, 1931]. When fed in the amounts used in this work *d*-acetylcysteine showed no signs of being poisonous and did not affect the dog's nitrogen excretion appreciably.

The results of nine experiments are as follow.

Dog	Dose g.	Route	% of dose excreted as sulphate in 2 days	% excreted as neutral sulphur	% of dose accounted for
1. Patsy	1.63	Orally	34	32	66
2. "	"	"	59	35	94
3. "	"	"	56	31	87
4. Christina	"	"	46	39	85
5. "	"	"	40	31	71
6. "	"	"	47	30	77
7. "	"	Subcutaneously	29	39	68
8. "	0.815	Orally	52	27	79
9. "	1.63	Subcutaneously	27	46	73

In Exps. 1, 3, 5 and 9 *d*-acetylcysteine was prepared by the old method and in the others by the ketene method.

The rise in neutral sulphur observed after feeding acetylcysteine is due, in part at least, to the excretion of that substance unaltered in the urine. From a mixed batch of urines passed on the day following the dose an amount of acetylcysteine corresponding to one-fifth of the rise in neutral sulphur was isolated. The method of isolation (precipitation with acid mercuric sulphate followed by the removal of sulphuric acid from the decomposed mercury precipitate and precipitation with lead acetate) could not, however, be expected to give even an approximately quantitative recovery.

A sample of each day's urine was reduced with zinc and sulphuric acid and titrated with *N*/100 iodine. The titration figure agreed closely with the rise in neutral sulphur on each dose day, but we feel that the actual value has little quantitative significance.

SUMMARY.

d-Acetylcysteine can be conveniently prepared by the action of ketene on cystine in alkaline solution.

When *d*-acetylcysteine is fed to a dog 48 % is excreted as sulphate and 32 % as neutral sulphur. It is less readily oxidised when given subcutaneously.

We thank the Government Grant Committee of the Royal Society for grants in aid of this research.

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CCXXXIV. THE DETERMINATION OF THE ANTINEURITIC VITAMIN.

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PART I. PIGEONS.

THE methods employed at the present time for the estimation of the antineuritic vitamin are varied. Pigeons have long been used in tests in which the vitamin is administered (a) to prevent head retraction, (b) to cure head retraction and (c) to maintain body weight. Recently Kline *et al.* [1932] have described the use of chicks. Rats have been used in growth tests and in tests in which paralytic symptoms are cured [Smith, 1930]. Mice have also been recommended by Freudenberg and Cerecedo [1931] for growth tests.

Since the number of different factors discovered to be present in the commoner sources of the antineuritic vitamin continues to increase, methods of estimation in which an antineuritic effect is produced have an advantage over tests depending on growth or weight maintenance. Further an element of doubt remains whether the principle which cures the symptoms of paralysis in rats is identical with the antineuritic principle of Eijkman. It is true that Smith [1930] found that concentrates prepared by Seidell which prevented the onset of symptoms in pigeons were able to cure the symptoms in rats, but the ratio between the pigeon maintenance dose and the curative rat dose was not the same in different preparations. We feel that the claim of a method, in which birds are not used, to measure the antineuritic vitamin must rest ultimately on evidence that results obtained by that method are parallel with results obtained by a method in which birds are used.

Tests on pigeons must in our opinion be curative tests; as Smith has pointed out, a preventive test such as that of Williams [1916; 1917] takes too long, and a test like that of Seidell [1922], in which maintenance of body weight is the criterion, cannot be assumed to be a test for the antineuritic vitamin. It has, however, been difficult to find a curative test which gives trustworthy results. Kinnersley *et al.* [1928] have discussed the curative test in detail and base their procedure on the view "that there is fundamentally a relation between the amount of the factor supplied and the time of protection after cure."

When a pigeon develops head retraction after being fed on polished rice, Kinnersley *et al.* administer a dose of the preparation to be tested; if no relief of symptoms is seen, they give a further dose; they observe the number of days for which the pigeon remains cured. If the total dose of the preparation administered is x g., and if the duration of the cure is y days, they express the

potency of the preparation as y/x "day doses per g." The average result from several birds is taken as the true result.

As the work in this laboratory involves many routine examinations, we have been attracted to the curative test for pigeons because of the simplicity of the test; it involves less work and skilled attention than the rat test described by Smith, or indeed than any other vitamin test. We have accordingly investigated the response of pigeons suffering from head retraction to different doses of several preparations containing vitamin B₁, testing the effect of each dose of a preparation upon several pigeons. The investigation has been continued during 31 months in which 688 birds have been given a diet of polished rice and water; as a result we are proposing a different method of using pigeons which we believe makes it possible for more accurate results to be obtained than are given by the day-dose method in routine tests.

Details of the treatment of the pigeons.

The pigeons were obtained from a dealer in groups of 30 at one time and were birds weighing 300–450 g. We did not give them a stock diet for a preliminary period but fed them on polished rice at once; nor did we wash the rice. Out of the total number of 688 birds used by us, head retraction occurred in 310 (45 %). The figures for different months are given in Table I and show

Table I. *Apparent seasonal variation in percentage of pigeons developing head retraction on a polished rice diet.*

	Total no. of birds given polished rice diet			Percentage of birds in which symptoms occurred in 30 days		
	1931	1932	1933	1931	1932	1933
January	30	—	32	23	—	19
February	30	32	32	40	42	69
March	23	32	30	40	53	60
April	32	30	—	50	47	—
May	32	24	30	41	37	57
June	32	—	—	56	—	—
July	20	—	24	45	—	37
August	—	—	—	—	—	—
September	31	—	—	55	—	—
October	32	32	—	53	47	—
November	32	32	—	59	34	—
December	32	32	—	28	42	—

that the proportion was lower in January, when the weather was cold. When head retraction was seen in a pigeon, the bird had usually lost from 70 to 90 g., though occasionally as little as 50 or as much as 110 g. Birds which did not show head retraction in 30 days were not used.

The pigeons were kept in the open air in cages with wire netting screens of $\frac{1}{2}$ -inch diameter to prevent access to faeces. When a pigeon showed head retraction, it was taken to the laboratory, the dose was given by mouth, and without delay it was put again in the open air in a separate cage. Attention was not paid to symptoms other than those of typical head retraction. The pigeon was then observed at intervals and its condition recorded at the end of each period of 24 hours from the time the dose was given. The pigeon was observed for several days to see for how long it remained free from symptoms. If head retraction had fully disappeared at the end of 24 hours, but had reappeared by 48 hours, the result was recorded as a cure for 1 day. Freedom

from symptoms for 48 hours, but for less than 72 hours, was recorded as a cure for 2 days, and so on.

If a pigeon was not cured within a few hours of the administration of the dose, no further dose was given. All doses were given in about 5 cc. of water, half of the water being kept to wash down traces. A pipette fitted with a short rubber tube of 2 mm. internal diameter was used.

Preparations examined.

The preparations examined were (1) a sample of dried yeast supplied by the National Institute for Medical Research for an investigation undertaken as a preliminary to the International Conference in London, 1931; (2) a sample of activated acid clay prepared by Prof. Jansen of Amsterdam and supplied by the National Institute for Medical Research; (3) a second sample of acid clay proposed for use as the International Standard of the antineuritic vitamin, also supplied by the National Institute for Medical Research; (4) two concentrated solutions kindly given to us by Prof. Peters; (5) a commercial extract of rice polishings; (6) a commercial soft extract of yeast.

These six preparations appeared to us to provide a sufficiently diverse assortment of preparations of the antineuritic vitamin.

EXPERIMENTAL RESULTS.

It will be convenient to describe some results relating to the method of estimating potency used by Kinnersley *et al.* As already stated, the percentage of pigeons which we have observed to develop head retraction within 30 days when fed on polished rice is 45. Prof. Peters has kindly informed us that the percentage observed in his laboratory is almost the same. We suggest that the close correspondence between these percentages means that certain precautions taken in Prof. Peters's laboratory are unnecessary (*e.g.* feeding the pigeons on a uniform stock diet for several days before giving them the rice diet and washing the rice). The omission of these steps means a saving of time and expense in routine work.

The relation between dose and duration of cure.

We have examined the conclusion of Kinnersley *et al.* that there is a relation between the amount of the antineuritic factor supplied and the time of protection after cure, and the results are given in Table II.

The preparations examined were each administered in one or more doses, and the average duration of cure was calculated for each selected dose. Thus the average duration of the cure of 11 birds to which 0.03 g. of the original sample of acid clay was administered was 3.7 days; hence by Peters's method the potency was 123 day doses per g. In calculating the average duration of cure, no account was taken of birds to which the dose was administered and which were not cured. The final column in Table II gives the potency of each substance expressed as units per g. or per cc., taking the unit as the activity present in 0.01 g. of the standard acid clay. The different figures for the potency of each substance should of course be alike if the day-dose method of comparing the activity of the different preparations with the standard is satisfactory. Inspection shows that the different figures are by no means alike. The potency of the original sample of acid clay was found to be 357, 176, 104 and 91 units per g. To the first of these figures less attention can be paid since it is based

Table II. *Vitamin B₁ potency of various substances estimated in terms of the International Standard by day-dose method.*

Material	Dose	No. of birds cured	Average duration of cure (days)	Day doses per g. or cc.	Potency in terms of Standard units per g. or cc.
Acid clay (original sample)	0.015 g.	4	3.75	250	357
	0.03 g.	11	3.7	123	176
	0.06 g.	16	4.4	73	104
	0.12 g.	13	7.7	64	91
Acid clay (Standard)	0.03 g.	7	2.1	70	100
Dried yeast	0.031 g.	2	4.5	145	207
	0.062 g.	5	1.8	28.8	41
	0.125 g.	15	4.4	35.2	50
	0.25 g.	16	5.9	23.6	34
Vitamin B ₁ solution (a)	0.1 cc.	4	2.0	20	29
	(b) 0.2 cc.	6	1.8	9	13
	(a) 0.25 cc.	7	2.1	8	11.4
	(b) 0.35 cc.	9	2.5	7	10
Extract of rice polishings	0.25 cc.	5	1.6	6.4	9
	0.5 cc.	10	5.1	10.2	14
	1.0 cc.	10	5.7	5.7	8
	2.0 cc.	11	7.5	3.75	5.3
Yeast (soft extract)	0.12 g.	3	1.7	14	20
	0.24 g.	6	3.5	14	20

Note. The vitamin B₁ solutions (a) and (b) were two different tubes kindly supplied by Prof. Peters, which were probably, but not certainly, alike in potency. Since examination showed no difference between them, they have been tabulated as one preparation.

on a cure of only 4 birds; but there is no explanation of the difference between the estimate of 176 units per g. and the other estimates of 104 and 91 units per g., save that the error of the method is large, or that the relationship between duration of cure and dose of vitamin B₁ given is not a straight line. (See the section on the statistical examination of these results.) The potency of the extract of rice polishings was found to be 9, 14, 8 and 5.3 units per g., the highest value being nearly three times the lowest.

In spite of these divergences there are some similarities; the two values 104 and 91 for the original sample of acid clay are close together; the highest and lowest of the three figures for dried yeast, 41, 50 and 34 do not differ by more than 50 %; the three figures 13, 11.4 and 10 for the vitamin B₁ solution are close together; two of the figures for the extract of rice polishings are respectively 8 and 9, while the two figures for the soft extract of yeast are identical.

We draw the conclusion that there is certainly foundation for the view of Kinnersley *et al.* that a relation exists between the size of the dose of vitamin B₁ administered and the duration of the cure, but that, when groups of 10 or 15 birds are used for an estimation, errors of 50 or 100 % may occur. It will be observed that the potency in day-doses per g. or per cc. of the materials in Table II diminished as the dose which was examined grew larger; it follows that the potency of any unknown material expressed as day doses per g. will depend to some extent on the dose which is chosen, becoming lower as the dose becomes higher. It is right to point out that these comments apply only to experiments in which different pigeons are used for examining the effect of different doses. If the same group of pigeons had been used for testing the effect of each of the doses of the different substances mentioned in Table II, it is possible that we would have found that the average duration of cure was proportional to the

dose; Kinnersley *et al.* [1928] have published some evidence in favour of this expectation. For routine purposes, when a preparation is to be compared with the International Standard, the use of the same group of pigeons for both standard and unknown preparations would necessitate a long delay in the completion of a test.

The percentage of birds cured.

Apart from the attempt of Kinnersley *et al.* to estimate potency in terms of "day-doses," other workers have determined potency by finding the minimum curative dose. The difficulty of doing this arises from the variation in the response of different pigeons; head retraction is not cured in some birds by a dose of the vitamin which will satisfactorily cure others, and in practice it is impossible to define what is meant by a "minimum curative dose." It is tempting to explain differences of this kind by supposing that at the time head retraction occurs the deficient diet has left one bird less able to absorb the vitamin than another; but such explanations do not help to overcome the practical difficulty of estimation. Similar variations in the response of animals to drugs are well known in pharmacology, and Trevan [1927] showed how they were to be overcome. We have followed the principles demonstrated by Trevan in the following experiment.

The sample of acid clay first supplied by the National Institute for Medical Research and the sample of dried yeast were tested in different doses to determine the proportion of birds cured by each dose. Thus the dose of 0.015 g. acid clay was given to each of 16 pigeons with head retraction; 4 were cured and remained cured for at least 24 hours; the dose of 0.03 g. acid clay was given to each of another group of 16 birds, of which 8 were cured. The results are recorded in Table III. It will be seen that as the dose either of acid clay or of dried yeast was increased, the percentage of birds cured also increased. In

Table III. *Vitamin B₁ potency of various substances estimated in terms of the I.S. by the percentage of birds cured.*

Material	Dose	No. of birds given dose	No. cured	% cured	Units per g. or cc.	Weighted mean (units)
Acid clay (original sample)	0.015 g.	16	4	25	118	110
	0.03 g.	23	11	48	100	
	0.06 g.	20	16	80	115	
	0.12 g.	20	13	65	—	
Acid clay (Standard)	0.03 g.	15	7	47	100	100
Dried yeast	0.031 g.	16	2	12.5	32.1	33.8
	0.062 g.	16	5	31	35.7	
	0.125 g.	20	15	75	39.8	
	0.250 g.	20	16	80	27.7	
Vitamin B ₁ solution (a)	0.1 cc.	13	4	31	22.1	16.2
	(b) 0.2 cc.	10	6	60	17.8	
	(a) 0.25 cc.	13	7	54	13.1	
	(b) 0.35 cc.	13	9	70	12.4	
Extract of rice polishings	0.25 cc.	8	5	62	14.8	17.5
	0.5 cc.	12	10	83	24.6	
	1.0 cc.	12	10	83	12.3	
	2.0 cc.	12	11	92	—	
Yeast (soft extract)	0.12 g.	10	3	30	17.9	19.6
	0.24 g.	8	6	75	20.7	

Fig. 1 the percentage of birds cured has been plotted against the dose, and the points for the acid clay and for the dried yeast fit curves of the usual sigmoid shape. The point indicating the result of administering the largest dose of acid clay lies off the curve through the other points; it was observed that all the birds which were not cured by this dose were dead on the day after the dose was given. This unusual occurrence gave us the impression that the curative action of the large dose was complicated by a toxic effect.

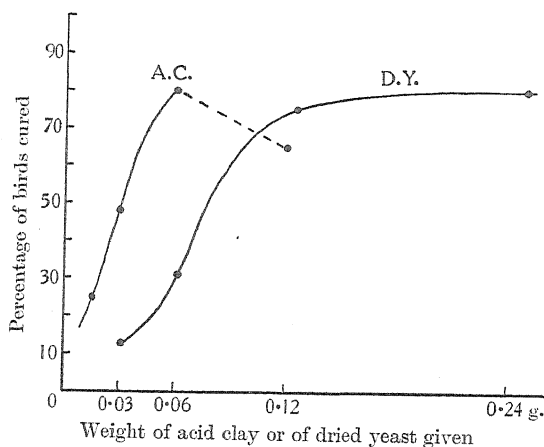


Fig. 1. A.C., Curve of response of polyneuritic pigeons to graded doses of activated acid clay (Jansen's preparation of vitamin B₁). D.Y., Curve of response of polyneuritic pigeons to graded doses of dried yeast.

From the two curves shown in Fig. 1 it is possible to make a quantitative comparison between the potency of the dried yeast and that of the acid clay. Thus a line parallel with the abscissa may be drawn to cut the curves at different points, and the ratio of the abscissae determined. Thus such a line drawn through the ordinate corresponding to 30 % cuts the acid clay curve at a point of which the abscissa is 0.02, and cuts the dried yeast curve at a point of which the abscissa is 0.06. The ratio of the potency of dried yeast to acid clay is, therefore, 0.02/0.06, or 0.33. Similarly the ratio of the potency determined by a line drawn through the ordinate corresponding to 50 % is 0.39, and that determined by a line drawn at 70 % is 0.42. The three figures 0.33, 0.39 and 0.42 give an average of 0.38.

The estimation of unknown preparations.

While it is true that a comparison of the potency of dried yeast with that of acid clay has been obtained by the foregoing experiments, it may be argued that the number of pigeons needed to construct a curve for an unknown preparation like the curve in Fig. 1 is too great for the method to be of practical value. The testing of unknown preparations can be abbreviated, however, in the following way. We have constructed from the two curves in Fig. 1 a third curve shown in Fig. 2, which is an approximation to the characteristic curve [see Trevan, 1927] for the response of pigeons with head retraction to doses of the antineuritic vitamin. The ordinates in Fig. 2 are, as in Fig. 1, the percentage of pigeons cured 24 hours after administration of the dose. The abscissae are doses expressed in arbitrary figures. The abscissa corresponding to the cure of

50 % of birds is given the value 1.0. Half-way between this point and the origin is 0.5, etc. The points on the acid clay curve in Fig. 1 are points corresponding to the cure of 25, 50 and 80 % of pigeons; if the dose curing 50 % be taken as 1.0, then the abscissae of these points in Fig. 2 are 0.5, 1.0 and 2.0, for the doses were in this proportion. Similarly the points on the dried yeast curve are plotted in Fig. 2 again taking the dose (determined by interpolation in

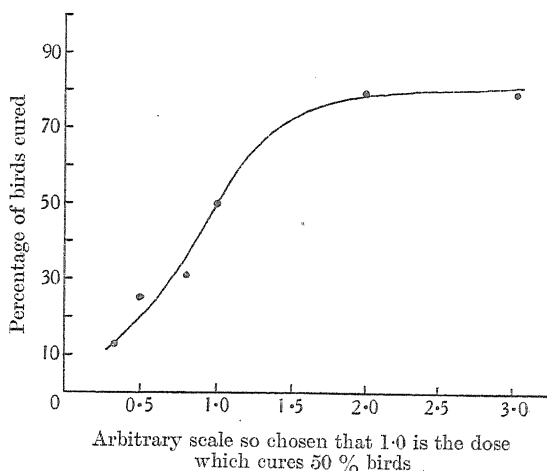


Fig. 2. Curve of response constructed from results of tests on acid clay and on dried yeast.

Fig. 1) which cures 50 % of pigeons as 1.0. The points on the two curves in Fig. 1, adjusted in this way, fit one curve remarkably well, the coincidence supporting the view that the same phenomenon is responsible for the shape of both curves in Fig. 1.

The curve in Fig. 2 can now be used for the estimation of unknown preparations of the vitamin, and the manner of its use is most simply explained by a comparison which was made of the two water clear solutions containing vitamin B₁, prepared by Prof. Peters, with the International Standard acid clay.

Comparison of vitamin B₁ solution with the Standard.

The vitamin B₁ solution was administered in a dose of 0.1 cc. to each of 13 birds suffering from head retraction; 4 birds were cured (approx. 30 %). The International Standard acid clay was administered to 15 birds suffering from head retraction in a dose of 0.03 g.; 7 birds were cured (approx. 50 %). The relative potency of doses curing 30 and 50 % of birds is given by Fig. 2 as 0.65 to 1.0.

$$\begin{aligned} \text{Hence} \quad \frac{0.1 \text{ cc. vitamin B}_1 \text{ solution}}{0.03 \text{ g. Standard}} &= \frac{0.65}{1.0} \\ \text{or} \quad 1 \text{ cc. vitamin B}_1 \text{ solution} &= 0.2 \text{ g. Standard} \\ &= 20 \text{ units.} \end{aligned}$$

A further examination of the vitamin B₁ solution was made in which 0.25 cc. was administered to each of 13 birds, of which 7 were cured, or approximately 50 %; hence 0.25 cc. vitamin B₁ solution was found approximately equivalent to 0.03 g. Standard, which contains by definition 3 units; hence 1 cc. = 12 units.

No further observations were possible with this material, as very little was left, but Prof. Peters kindly sent us another tube of solution which he believed to be similar to the first tube, though he was not certain. The small remainder of the first tube was mixed with this second tube, and the mixture was administered to pigeons in the two doses 0.2 and 0.35 cc. The first dose cured 6 out of 10 birds, indicating a potency of 17.5 units per cc., and the second dose cured 9 out of 13 birds, indicating a potency of 12 units per cc.

Comparison of the day-dose and percentage-cure methods.

The results obtained with the two solutions supplied by Prof. Peters made possible a useful comparison shown in Table IV of the day-dose method of calculating potency and the percentage-cure method. For this comparison the

Table IV. *Examination of vitamin B₁ solution.*

First tube, A; second tube, B.

Dose (cc.)	Day-dose method	Units per cc.		
		Average	Percentage- cure method	Average
0.1 A	29	15.9	20	15.4
0.2 B	13		17.5	
0.25 A	11.6		12	
0.35 B	10		12	

two solutions examined were assumed to be identical in potency; the results indicated that they were. The results by the day-dose method have already been given in Table II.

Observations on other preparations.

The extract of rice polishings and the soft extract of yeast were also examined by the percentage-cure method. In a dose of 0.25 cc. the extract of rice polishings cured 5 out of 8 pigeons; in a dose of 0.12 g. the soft extract of yeast cured 3 out of 10 pigeons, while a dose of 0.24 g. cured 6 out of 8 pigeons. From these results the potency in units per cc. or per g. has been calculated and expressed in Table V.

Table V. *Comparison between results obtained by the day-dose and percentage-cure methods.*

Material	Dose	Potency by day-dose method (units per g. or cc.)	Potency by per- centage-cure method (units per g. or cc.)
Acid clay (original sample)	0.03 g.	176	123
	0.06 g.	104	
	0.12 g.	91	
Dried yeast	0.062 g.	41	42
	0.125 g.	50	
	0.25 g.	34	
Extract of rice polishings	0.25 cc.	10.8	12
Yeast (soft extract)	0.12 g.	20	17
	0.24 g.	21	

The agreement seen in Table V between the average figures for the potency by the day-dose method and for the potency by the percentage-cure method is striking; for the original sample of acid clay the agreement would be close

but for the one high estimation by the day-dose method. The average figures for the two methods differ by 20 % for dried yeast, by 11 % for the extract of rice polishings, while the means of the two pairs of results for the soft extract of yeast differ by 10 %. The two methods have given the same average results in these experiments because several doses were tested, and the high values calculated from the low doses in the day-dose method balanced the low values calculated from the high doses.

Non-specific cures.

The pigeon curative method has been criticised because some other substances than the antineuritic vitamin will give temporary relief from head retraction in some birds.

Two questions appear to us to be confused in the minds of those who offer this criticism, namely whether a substance contains any vitamin B₁, and how much vitamin B₁ it contains. In detecting the presence of any vitamin B₁, it is obviously important to watch for non-specific effects; on the other hand, in determining the potency of a substance known to contain vitamin B₁, it is less important, since the substances which produce non-specific cures can usually be assumed to be absent. Histamine, pilocarpine, nitrites, thyroxine and choline [Dutcher, 1919; Abderhalden, 1923; Peters, 1924] are not present in extracts of yeast or rice polishings in sufficient quantity to interfere with the estimation of the vitamin B₁.

Peters adopts the precaution of administering dextrose to each bird which develops head retraction; we have, therefore, examined the effect of giving 0.05 g. dextrose in 5 cc. water to a series of pigeons prepared here. Out of 16 birds to which the dose was administered, 2 were cured for periods of 24 and 48 hours respectively. It follows from this result, that if we were examining a substance of unknown origin, in which dextrose might be present, it would be wrong to calculate the amount of vitamin from the cure of only a small proportion of birds; it would be necessary to test the effect of a larger dose. It is of course wise to do this whatever the origin of the preparation being tested; thus, in testing the soft extract of yeast as already described, the potency was first calculated from the cure of 3 birds out of 10 by a dose of 0.12 g.; a further estimate was then made by administering twice the dose, when 6 out of 8 birds were cured. The final estimate of 18.2 units per g. did not differ greatly from the first estimate of 16.6 units per g.

Birds cured for 1 day.

The definition of a cure of head retraction given earlier in the paper was that a bird should be found free from symptoms at the end of 24 hours after the dose was administered. If a bird found to be cured at that time developed symptoms again 48 hours after the first sign of head retraction, the cure was counted as a cure for 1 day. The opinion was expressed to us that it was unsafe to assume that a cure for less than 48 hours was a genuine cure. We have, therefore, recalculated our results, excluding all birds in which the cure lasted for less than 48 hours. We have found, however, that the results so obtained did not differ appreciably from those already given in Tables II and III. Thus the potency of dried yeast became 47 units per g. instead of 42 units per g. when using the day-dose method of comparison and became 34 units per g. instead of 38 units per g. using the percentage-cure method.

Duration of head retraction before dosing.

When pigeons developed head retraction on the diet of rice, some were observed at the beginning of the day, and some were observed later; as no inspections were made during the night, birds found with head retraction in the morning may have had the symptoms for 15 hours before the dose was given, whereas birds found later in the day were given the dose within an hour or two of the onset. We have examined the records to see whether the birds in a group which were not cured by a particular dose were those which were found with symptoms in the early morning, and whether the birds which were cured were those found later. The examination showed no such correlation; the cure of head retraction by a particular dose was not dependent on the lapse of a short time between the onset of symptoms and the administration.

PART II. RATS.

THE GROWTH-PROMOTING PROPERTY OF VITAMIN B₁.

It has been shown by many workers that vitamin B₁ (as distinct from vitamin B₂ or the vitamin B complex) is necessary for the growth of the rat. Sherman and Axtmayer [1927] demonstrated the supplementary nature of wheat germ and dried milk and concluded that this was due to the greater amount of vitamin B₁ in wheat germ than in dried milk. Chick and Roscoe [1927] found that an extract of the antineuritic vitamin prepared from yeast according to Peters's method would not promote growth in rats fed on a diet deficient in vitamin B (complex). If, however, this was supplemented by a daily dose of yeast autoclaved at 120° for 5 hours to destroy the antineuritic vitamin, growth was resumed. They showed also that rats lose weight and die more quickly on a shortage of vitamin B₁ than on a shortage of vitamin B₂. Hassan and Drummond [1927] showed that two factors in yeast, differentiated by their behaviour to alkalis, were necessary for the normal rate of growth of rats fed on high-protein diets. It then became generally recognised that, in order to estimate vitamin B₁ by means of its growth-promoting property, vitamin B₂ must be liberally supplied to the rats.

Aykroyd and Roscoe [1929], in estimations of vitamin B₂, gave a daily dose of 0.1 cc. of Peters's antineuritic concentrate (equivalent to 0.6 g. yeast) to their rats in addition to the vitamin B-free diet.

Finally, Chick and Roscoe [1929] developed a method for the estimation of vitamin B₁ in foodstuffs by means of its growth-promoting property. They supplied vitamin B₂ by a daily dose of autoclaved yeast (120° for 5 hours) or substituted the purified caseinogen of the diet by coagulated egg-white from fresh eggs, which they had shown to contain vitamin B₂. They discarded the latter method, however, as the growth of the rats was not maintained long enough for the test, and they concluded that autoclaved yeast contains a heat-stable factor necessary for growth and distinct from both vitamin B₁ and vitamin B₂. Fresh egg-white does not contain this factor.

Roscoe [1930; 1931, 1, 2] compared the vitamin B₁ content of various vegetables and fruits by determining the dry weight of each necessary to produce a given rate of growth in 5 weeks. Vitamin B₂ was given to each rat as a daily dose (equivalent to 0.5 g. dry yeast) of an extract made from washed brewer's yeast by boiling it with 0.01 % acetic acid and then autoclaving the extract for 5 hours at 120° (*p_H* ca. 5.0).

Halliday [1932] supplied vitamin B₂ as 15 % autoclaved yeast in the basal diet of rats which were used for estimations of vitamin B₁.

Guerrant and Dutcher [1932] used as a source of vitamin B₂ a dry autoclaved (6 hours, 15 lbs.) extract of yeast which had been precipitated at an 80 % alcohol concentration of the aqueous extract in the filtrate from 50 % alcohol concentration.

Chick and Jackson [1932], in estimating vitamin B₁ with the rat as the experimental animal, used as a source of B₂ a small daily ration of a watery yeast extract, autoclaved for 4 hours at 120° at p_H 5.0.

It then seemed to us that it would be useful to construct a curve of response relating average increase in weight of groups of rats to daily dose of vitamin B₁ given, as we had previously done for vitamin A and vitamin D.

I. Diet of the rats.

Young rats weighing 55–70 g. were given a diet consisting of:

	%
Caseinogen (light white B.D.H. not extracted) ...	15
Dextrinised rice starch	71
Agar-agar	2
Salt mixture (Steenbock's 40)	4
Autoclaved yeast... ..	8

Each rat was given 5 drops of a good sample of cod-liver oil twice a week to supply vitamins A and D.

The yeast was spread on tins in thin layers, 40 g. over 44 sq. in., and autoclaved for 6 hours at 15 lbs. additional pressure. It became dark brown in colour and moist. It was dried by leaving overnight in an electric oven whose current had been turned on until the temperature was raised to 100° and then turned off before the trays of yeast were put into it. The yeast was caked after drying and had to be ground in a mortar.

The rats were given this diet until they ceased to grow. During this preparatory period of 14 to 18 days, nearly every rat increased in weight by at least 10 g., many by 20 g. and some even by 30 g. Those which were given no vitamin B₁ after growth ceased, lost weight rapidly and died in 21–38 days from the beginning of the preparatory period.

The rats were kept 4 together in a cage during the preparatory period, on grids of 0.3 in. mesh. During the giving of doses, each rat was kept in a separate cage on a grid of 0.5 in. mesh.

Twelve litters of 5 or 6 rats each were used for testing 5–6 doses of acid clay, each rat of a litter receiving a different dose of acid clay. The doses tested were 0, 0.005, 0.01, 0.02, 0.04, 0.1 g. For the first 6 litters each dose was weighed daily and mixed with a little water in a separate dish for each rat. Some of the rats refused to eat their doses completely but when the dose was mixed with a little dextrin it was always eaten. For the later work, the acid clay was mixed at once with dextrin (1 part acid clay to 4 or to 9 parts dextrin as convenient) and the diluted material used for all the rats.

II. First curve of response to graded doses of activated acid clay, the International Standard of reference for vitamin B₁ (8 % autoclaved yeast in the diet).

No rats developed polyneuritis or convulsions [Smith, 1930] during this experiment, whether they received a dose of acid clay or not.

The growth response of the rats was graded to the dose of acid clay given. The male rats made greater increases in weight on each dose (but one) than

the female rats (Fig. 3), but the difference was less than the difference in similar experiments with vitamins A and D [Coward *et al.*, 1931; 1932]. The mean increases in weight of the bucks and does respectively have themselves been averaged for constructing a curve of response for use in interpreting estimations on other substances. The results of the tests on other substances have been treated similarly for the sake of simplicity.

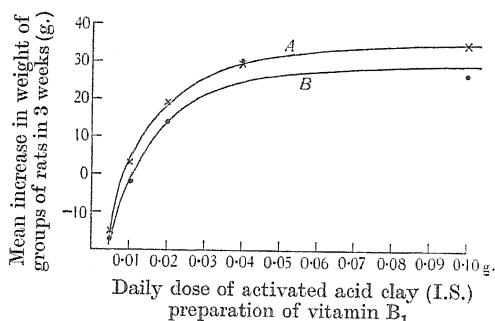


Fig. 3. Curves of response of groups of rats to different doses of activated acid clay (I.S. for vitamin B₁ estimations). Each rat of a group received the same daily dose for 3 weeks. The basal diet contained only 8 % autoclaved yeast as a source of vitamin B₂.

A, determinations from male rats.

B, determinations from female rats.

In the first 5 litters we used for the construction of this curve, several of the rats given the highest dose of acid clay (0.04 g.) grew less quickly than was expected from the growth of the rats on the lower doses. We, therefore, used one rat of each of the later litters for testing a dose of 0.1 g. acid clay. The response of the bucks to this dose was higher than to the dose of 0.04 g. but that of the does was slightly less. This, together with results of tests of other substances made at the same time, led us to the conclusion that the basal diet we were using was deficient in some substance or substances other than vitamin B₁. We suspected the destruction of vitamin B₄ in the autoclaving of the dried yeast. We were able to examine this possibility through the kindness of Dr V. Walker (Reader) who gave us sufficient solution of vitamin B₄ to dose 12 of our rats receiving different doses of acid clay, but growing very slowly. This did not, however, increase the rate of growth of any one of our rats, and we concluded that the diet was not lacking in vitamin B₄. We then suspected a deficiency of vitamin B₂ or some other heat-labile factor in the basal diet. Tests on other substances (Section III) and the construction of a fresh curve from rats given a basal diet containing 20 % autoclaved yeast (Section IV) confirmed this view.

III. Comparison of other substances with activated acid clay with regard to their vitamin B₁ potency.

Four substances were examined for their content of vitamin B₁ at the same time that the curve of response to doses of activated acid clay was being constructed. The substances were (a) a sample of dried yeast (used by several laboratories in connection with preliminary work on the International Standard), (b) a sample of wheat embryo (also used by the same laboratories for the same work), (c) a commercial sample of a food and (d) a commercial sample

of a yeast extract (I). Each was tested in two different doses. It happened that the lower dose of each substance gave a result comparable with the result on a low dose of acid clay, but the higher dose of each substance gave a result considerably higher than that given by the highest dose of acid clay. The mean increase in weight produced by each of the higher doses was well above the curve. In Fig. 4 the mean increase in weight of the animals given the lower

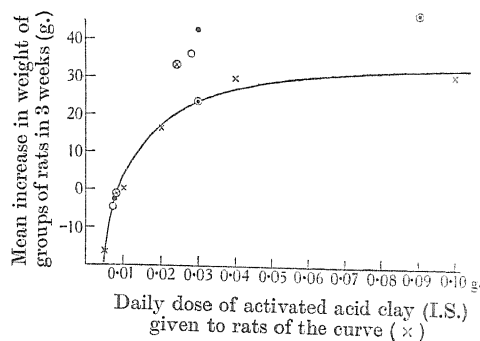


Fig. 4. Uselessness of this curve of response to estimate the vitamin B₁ content of other substances. (Each point is the average of the mean increases in weight of the bucks and does respectively, i.e. no point is overweighted by an excess of results from bucks.)

- × = Mean increases in weight of the groups of rats given graded doses of the Standard.
- = Mean increases in weight of groups of rats given daily 0.05 and 0.2 g. respectively of a sample of dried yeast I.
- ⊙ = Mean increases in weight of groups of rats given daily 1.0 and 3.0 g. respectively of a commercial food sample.
- ⊗ = Mean increases in weight of groups of rats given daily 0.1 and 0.3 g. respectively of a sample of wheat embryo.
- = Mean increases in weight of groups of rats given daily 0.05 and 0.2 g. respectively of yeast extract I.

The result from the lower dose of each test was plotted on the curve itself. The result from the higher dose of each test was plotted against the abscissa corresponding to the appropriate multiple of the abscissa found for the lower dose.

dose of the substance was marked on the curve, the abscissa of that point noted, and the mean increase in weight of the animals given the higher dose was plotted against the abscissa which bore the same ratio to the first abscissa that the higher dose bore to the lower dose of substance tested. It was obvious that the dried yeast, the wheat embryo, the yeast extract and the food substance each contained some substance necessary for growth which was not supplied in sufficient amounts by our basal diet *plus* large doses of acid clay.

We, therefore, decided to repeat the experiment using 20 % autoclaved yeast in the diet which was then made up as follows.

	Parts
Caseinogen (light white, B.D.H. untreated) ...	15
Dextrinised rice starch ...	79
Agar-agar ...	2
Salt mixture (Steenbock's 40) ...	4
Autoclaved yeast ...	25

The same amount of cod-liver oil was given to each rat as before.

IV. *Second curve of response relating mean increase in weight of groups of rats to dose of vitamin B₁ given (20 % autoclaved yeast in basal diet).*

A second curve of response was constructed by the same method as the first, but 20 % yeast autoclaved under apparently the same conditions was used instead of 8 %. Four litters of rats were used for this experiment. The curve of response was steeper than the first (Fig. 5). It justified our conclusion that in the former experiment we had not given a large enough percentage of autoclaved yeast in the basal diet.

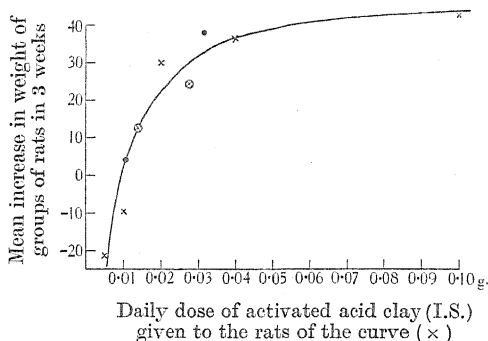


Fig. 5. Curve of response of groups of rats to graded doses of activated acid clay (I.S. for vitamin B₁ estimations). Each rat of a group received the same daily dose for 3 weeks. The basal diet contained 20 % autoclaved yeast as a source of vitamin B₂.

Usefulness of this curve for estimating the vitamin B₁ content of other substances.

x = Mean increases in weight of groups of rats given graded doses of the Standard.

• = Mean increases in weight of groups of rats given daily 0.05 and 0.15 g. respectively of dried yeast.

⊗ = Mean increases in weight of groups of rats given daily 0.1 and 0.2 g. respectively of a yeast extract II.

The results were plotted as in Fig. 4.

V. *Confirmation of the validity of the second curve of response relating mean increase in weight of groups of rats to dose of vitamin B₁ given.*

Two substances, dried yeast II and a yeast extract II (both different from those used previously), were tested in 2 doses each. The mean increases in weight of the 2 groups of rats given the lower doses of the 2 substances were plotted on the curve, and the mean increases in weight of the rats given the higher doses were plotted against the abscissae which bore the same ratios to the abscissae of the lower doses as the ratios of the actual doses. These points fell very nearly on the curve of response, one somewhat above the curve, the other somewhat below (Fig. 5). Thus the second curve appears to be a better one than the first in that, so far as it has been tested, the responses do not seem to be limited by an insufficiency of any factor in the basal diet. The response to a test substance when this diet is used appears to be a response to vitamin B₁ only.

VI. *The possible logarithmic nature of the curves.*

Each of the two curves described in this paper was constructed from 5 determinations of the mean increases in weight in 3 weeks of 5 groups of rats, the rats of each group being given daily doses of 0.005, 0.01, 0.02, 0.04 and 0.1 g. International Standard preparation of vitamin B₁. The curves were drawn as smoothly as possible near the points plotted on graph paper. Neither of the

curves so drawn was logarithmic, and the best logarithmic curve through either set of 5 points (determined from the best straight line through the points obtained by plotting mean increases against the logs of the doses) did not fit the actual points at all well. But a logarithmic curve fitted the four lower points of the first set very well and another fitted those of the second set only rather less well (Fig. 6). This is similar to our finding in our vitamin A work, in which

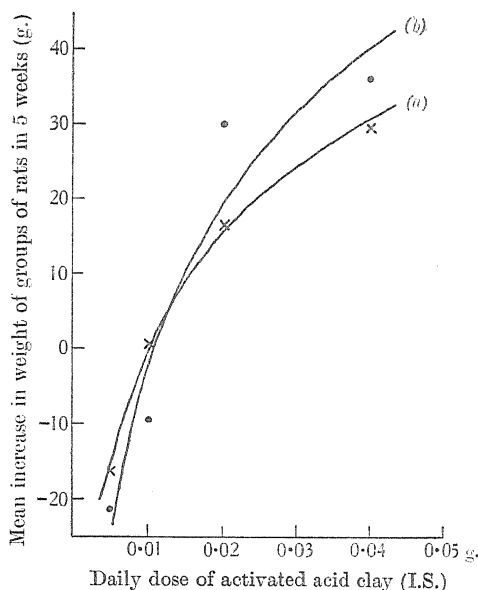


Fig. 6. The best logarithmic curves relating the mean increase in weight of groups of rats to dose of vitamin B₁ given when the basal diet contains (a) 8% and (b) 20% respectively of autoclaved yeast as a source of vitamin B₂.

a logarithmic curve fitted the responses of rats (mean increases in weight in 5 weeks) to doses of 0.25, 1.0, 1.5, 2.5, 7.5 mg. respectively of cod-liver oil, but not the response to the highest dose, 20.0 mg. It should, however, be safe to accept the logarithmic equations for the curves for the range of mean increases which determined the shapes of the curves.

The equation for the first vitamin B₁ curve reported in this paper (Fig. 6 (a)) for the range -16 to +30 g. mean increase in weight is $y = 102.3 + 51.1 \log x$.

The equation for the second curve reported in this paper (Fig. 6 (b)) for the range -21 to +36 g. mean increase in weight is $y = 139.4 + 70.4 \log x$.

VII. Justification for the acceptance of the logarithmic equations for the curves of response.

The logarithmic curve through the first 4 points in the first experiment fits those points so well that it can be accepted without question as representing the relation between response and dose of vitamin B₁ given for the range of doses 0.005 to 0.04 g. I.S. Its equation is $y = 102.3 + 51.1 \log x$.

The logarithmic curve through the first 4 points in the second experiment fits those points less well and it might be accepted with some hesitation. When, however, the results of feeding 2 different doses of each of 2 different substances were interpreted by means of this curve, the ratios of the apparent potencies

of the respective pairs of doses were so nearly the known ratios of the doses that we feel no hesitation in accepting the curve with its logarithmic equation as representing the relation between the mean increase in weight of a group of rats (all given the same dose) and the dose of vitamin B₁ given (Table VI). Its equation is $y = 139.4 + 70.4 \log x$.

Table VI. *Confirmation of validity of second curve of response relating mean increase in weight in 3 weeks of groups of rats to dose of vitamin B₁ given (I.S.).*

Substance tested	Daily dose (g.)	No. of rats	Mean increase in wt. in 3 weeks (g.)	Abscissa of curve corresponding to the increase in weight	Ratio of potencies of doses according to ratio of abscissae	Actual ratio of doses
Dried yeast II	0.05	3	4.2	0.0125	1:2.9	1:3
"	0.15	4	38.0	0.0365		
Yeast extract II	0.1	4	12.8	0.0160	1:1.6	1:2
"	0.2	4	24.5	0.0255		

VIII. *Interpretation, by means of the second curve, of results which could not be interpreted by means of the first curve.*

In Table VII are collected details of the tests of the four substances mentioned in Section III, which could not be interpreted by means of the curve of

Table VII. *Interpretation, by means of the second curve, of results which could not be interpreted by means of the first curve.*

Substance tested	Dose (g.)	No. of rats		Mean increase in wt. in 3 weeks (g.)		Average mean increase of ♂ and ♀ rats	Interpretation by second curve of response. Diet contained 20 % autoclaved yeast		
							Abscissa corresponding to mean increase in weight	Apparent ratio of doses	Actual ratio of doses
		♂	♀	♂	♀				
Dried yeast I	0.05	6	4	-1.3	-4	-2.65	0.0096	1:4.2	1:4
	0.2	7	3	49.7	36.0	42.85	0.0407		
Food substance	1.0	3	3	23.3	23.7	23.5	0.0226	1:2.1	1:3
	3.0	3	3	55.3	37.6	46.45	0.0467		
Wheat embryo	0.1	4	2	0.75	-3.0	-1.12	0.0101	1:3.5	1:3
	0.3	2	4	39.5	27.25	33.37	0.0352		
Yeast extract I	0.05	3	2	-6.7	-2.5	-4.6	0.0090	1:3.8	1:4
	0.2	3	2	39.3	33.0	36.15	0.0341		

response constructed about the same time and with the use of the same basal diet (containing 8 % autoclaved yeast). When these are interpreted by means of the second curve of response (basal diet containing 20 % autoclaved yeast), the ratios of the apparent vitamin B₁ potencies of the respective pairs of doses are found to be in very good agreement with the known ratios of the doses given.

This may be taken as good evidence that the basal diet which contained only 8 % autoclaved yeast was deficient in some factor necessary for growth which was not supplied by the activated acid clay but which was supplied by each of the other substances examined.

The potency of all substances examined has, therefore, been calculated by means of the second curve and stated in terms of the I.S. for vitamin B₁ in Table VIII.

Table VIII. *Potency of substances tested by the rat method in terms of the I.S. for vitamin B₁.*

Substance tested	Dose (g.)	Abscissa corresponding to mean increase in weight	No. of units vitamin B ₁ per g.
Dried yeast I	0.05	0.0096	19.2
	0.2	0.0407	20.35
Food substance	1.0	0.0226	2.26
	3.0	0.0467	1.56
Wheat embryo	0.1	0.0101	10.01
	0.3	0.0352	11.73
Yeast extract I	0.05	0.0090	18.0
	0.2	0.0341	17.05
Dried yeast II	0.05	0.0125	25.0
	0.15	0.0365	24.4
Yeast extract II	0.01	0.0160	16.0
	0.2	0.0255	12.8

DISCUSSION.

The point that appears to us of the greatest interest in this examination of the growth response of rats to graded doses of vitamin B₁ is that the slopes of the two curves of response obtained with 8 and 20 % respectively of autoclaved yeast in the basal diet are different. One might have expected the two curves to be superposed at their lower ends and the curve obtained with 8 % autoclaved yeast to bend away suddenly from the other one at the point where the maximum effect allowed by 8 % autoclaved yeast was reached. This, however, is not what happens. There is no sudden limiting of the response to vitamin B₁ when the supply of vitamin B₂ is insufficient. The inadequacy of the supply of vitamin B₂ affects the response to lower doses of vitamin B₁ as well as to higher doses. Thus, with only 8 % autoclaved yeast in the basal diet, a dose of some substance containing vitamin B₂ as well as vitamin B₁ would give a higher result than the same amount of vitamin B₁ in a substance free from vitamin B₂; provided the results fell on the curve, no error would be suspected (*cf.* our results depicted in Fig. 4) and the estimation of vitamin B₁ would be too high. This is not only of practical importance, but it seems to be of theoretical importance also as indicating an interdependence of the vitamins.

The difference in slope of the curves seems to indicate that the more vitamin B₂ (or some other factor, not B₂ or B₁, in the autoclaved yeast) in the basal diet, the greater is the response of rats to doses of vitamin B₁. Below the point of intersection (5 g. mean increase, 0.0125 g. Standard), the reverse appears to be true.

This is a similar result to the one reported by us [Coward *et al.*, 1932] concerning the dependence of the response to vitamin D on the amount of vitamin A in the basal diet (or given daily). We found that the larger the dose of carotene given to all rats daily, the greater was the slope of the curve of response to graded doses of vitamin D.

We do not conclude from these experiments that 20 % yeast autoclaved at 120° for 5 hours will always provide sufficient of the B vitamins (other than B₁)

to support normal growth. Indeed we have evidence (to be reported in a later paper) that 20 % of yeast autoclaved apparently in the same way may be far too small an amount for normal growth. We consider that our results demonstrate afresh the need for making a simultaneous test on at least one dose and preferably on two doses of a standard of reference whenever an examination of the vitamin B₁ content of a substance is made. The test can only be considered satisfactory if there is no evidence (such as we described in Section III) that the substance under test is supplying some factor other than vitamin B₁ which is not contained in sufficient amount in the basal diet.

PART III. THE VITAMIN B₁ POTENCY OF A SUBSTANCE AS ESTIMATED BY MEANS OF (a) PIGEONS AND (b) RATS.

The decision whether the tests by means of pigeons and the tests by means of rats measure the same factor rests on the results obtained from the examination of substances by both the methods. If both methods give the same result it may be assumed that the two methods measure the same factor. Two substances have been examined by both methods: (a) a soft extract of yeast I and (b) a sample of dried yeast I (Table IX).

Table IX.

Substance tested	Potency of substances as determined by tests on		
	(a) pigeons		(b) rats
	as calculated by day-dose method (units)	as calculated from % birds cured (units)	as calculated from mean increase in weight in 3 weeks (units)
Soft extract of yeast I	20.5	18.5	17.6
Dried yeast I	42.0	34.0	19.8
Dried yeast III	113.0	83.0	37.8

There is good agreement between the results of the tests of the soft yeast extract obtained by all three methods, but the pigeon test of the dried yeast makes it appear to be twice as potent as does the rat test. The chance that this divergence is due to the inaccuracy which is inevitable in all three tests has been calculated as about 1 in 4000, which is exceedingly small. It appears therefore, that for dried yeast, pigeons give a higher value for the vitamin B₁ potency than do rats. Confirmation of this result has recently been obtained by the examination of a third sample of dried yeast (III, Table IX)¹. The day-dose method gives results which vary according to the size of dose given as shown in Parts I and IV of this paper, therefore very little attention need be given to the result obtained by this method of calculating the potency of the dried yeast. The result from the percentage of birds cured, however, confirms the result obtained with the first sample of dried yeast; *viz.* the pigeon method of calculating the vitamin B₁ potency of dried yeast gives a higher figure than the rat method. Actually the potency obtained by the pigeon method is about double of that obtained by the rat method.

¹ This test was made in the course of a comparison of different workers' methods of estimation of vitamin B₁ arranged by Prof. A. Jung, University of Basel.

It appears, therefore, that there is some reason for thinking that the rat is less able to respond to the vitamin B₁ contained in dried yeast than is the pigeon. On the other hand, the rat gave the same result as did the pigeon for the soft extract of yeast I, and from this it must be concluded that the rat and pigeon can respond equally well to the vitamin B₁ in such an extract and also to that in the acid clay. Thus the rat-growth method may prove to be unsuitable for estimating the B₁ potency of some substances, but, on the other hand, polyneuritic pigeons are obviously not suitable for estimating the vitamin B₁ potency of bulky substances such as wheat germ or wholemeal bread. The pigeon test moreover has a probable error of +31 or -26 % when 9 birds are used for a single dose of a substance, whereas the rat test has a probable error of only +6.1 to -5.5 % when 9 animals are used. It must be recognised, however, that the pigeon test is one in which the estimation is based on a specific reaction to vitamin B₁ and it must, therefore, on general principles, be preferable to a growth test.

PART IV. FURTHER CONCLUSIONS DRAWN FROM A STATISTICAL EXAMINATION OF THE RESULTS.

I. PIGEONS.

A. METHOD OF CALCULATING RESULTS BY DURATION OF CURE.

(a) *Standard deviation of the average duration of cure.*

The mean variance of the number of days of the pigeons' cure was calculated from the 19 tests recorded in Table II by the formula $\sigma^2 = \frac{\sum d^2}{N - m}$ where N = the number of observations (*i.e.* the number of pigeons) and m = the number of means (*i.e.* the number of tests). It was found that

$$\sigma^2 = 9.02,$$

whence

$$\sigma = 3.$$

The number of pigeons from which the calculation was made is admittedly small, but it is large enough to use the value of σ found as a basis for certain calculations.

(1) *The standard deviation of the duration of the cure in relation to the duration of the cure.* It was to be doubted whether the variation in length of cure would be the same for pigeons given small doses as for pigeons given large doses of vitamin B₁. The results were, therefore, divided into 3 groups according to the average number of days of cure of the pigeons of the groups. The mean variance for each group was calculated and from it the standard deviation (Table X).

Table X.

No. of pigeons in group	Average no. of days cured	Standard deviation of mean
46	2.0	1.2
54	4.1	3.03
60	6.4	3.75

Thus there seems to be some evidence that there is less variation in the results obtained with low doses of vitamin B₁ than in those obtained with high doses.

(2) *Comparison between solids and liquids with regard to the pigeon's power of extracting and absorbing vitamin B₁ from them.* It might be expected that a pigeon in an abnormal state of health would be less able to utilise the vitamin B₁ in a solid preparation such as dried yeast or even activated acid clay than that in a liquid preparation such as Peters's concentrate, the liquid extract of rice polishings or the soft extract of yeast which was diluted with water before being given to the bird. Calculations were, therefore, made of the mean variances found in the tests on the liquid and solid preparations respectively. The values of σ ($= \sqrt{\text{mean variance}}$) of the two groups were found to be 2.93 and 3.06. These cannot be regarded as different. As the average duration of a cure of all the pigeons given a liquid preparation was 3.96 and that of all the pigeons given a solid preparation was 4.60 days it is evident that the similarity in the values of σ for the two groups has not arisen through a difference in absorbability being balanced by a difference in the average duration of cures of the two groups. Thus it must be concluded that pigeons such as were used in these tests can absorb vitamin B₁ from liquids and from solids such as the I.S. and dried yeast equally well.

(b) *Relation of duration of cure to dose of vitamin B₁ given.*

A curve has been constructed relating the duration of cure to dose of vitamin B₁ given. The data obtained from the doses of acid clay alone were not considered sufficient for constructing a curve for general application. Therefore, the acid clay equivalent of each dose of substance tested was calculated from the average value of that substance stated in Table III. The results were then averaged in 6 groups according as the dose given was (a) 0.011 to 0.016, (b) 0.021 to 0.024, (c) 0.030 to 0.032, (d) 0.040 to 0.048, (e) 0.056 to 0.060, (f) 0.085 to 0.087, (g) 0.12 to 0.175, (h) 0.35 g. of acid clay or its equivalent, and plotted as a curve relating average duration of cure to dose of acid clay (I.S. for vitamin B₁) (Fig. 7, continuous line). This curve is not logarithmic.

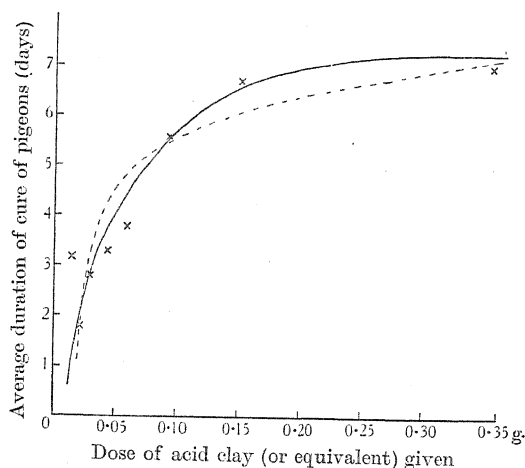


Fig. 7. Curve of response constructed from the duration of cures of polyneuritic pigeons given graded doses of activated acid clay or the equivalent of these doses in other substances. Continuous line—best curve drawn through points obtained experimentally. Broken line—best logarithmic curve through these points, but not adopted as it fits the points much less well than the other curve.

The best logarithmic curve through the points has been drawn as a broken line. It does not fit the points as well as the first curve drawn and there seems to be no reason for expecting the curve to be logarithmic; it has not been used.

(c) *The degree of accuracy obtainable by this method.*

The probable error of this test as obtained under the conditions described for this laboratory has been calculated in Table XI.

Table XI. *The probabilities of obtaining certain degrees of accuracy in this test. Standard error of duration of cure when 9 birds are used in a test.*

$$\epsilon = \frac{\sigma}{\sqrt{n}} = \frac{3}{\sqrt{9}} = 1.$$

Average duration of cure, say,	Abscissa corresponding to duration of cure	Range of duration of cure	Range of abscissae* corresponding to range of cure	% range of abscissae
4 days	0.049	(a) $\pm \frac{2}{3}\epsilon = \pm 0.67$ ($\frac{1}{2}$ chance)		
		4.67	0.064	130.6
		3.33	0.036	73.5
		(b) $\pm \epsilon = \pm 1.0$ ($\frac{2}{3}$ chance)		
		5.0	0.074	151.0
		3.0	0.032	65.3
		(c) $\pm 2\epsilon = \pm 2.0$ ($\frac{1}{2}$ chance)		
		6.0	0.110	224.5
		2.0	0.022	44.9

* Obtained by direct reading from curve (continuous line).

Thus the probable error of an estimation of vitamin B₁ by the use of a curve relating duration of cure to dose of vitamin B₁ given and using 9 pigeons for one dose of the substance under examination is about +31 or -26 %. We have not yet sufficient evidence to say whether a simultaneous test on the Standard should be made with every test of an unknown substance. On general principles it would seem to be desirable. The probable error of the estimation of the unknown in terms of the Standard then becomes (+31 or -26) $\times \sqrt{2}$ which becomes +43.7 or -37.0 %.

It is obvious from the shape of this curve of response that, as pointed out in the early part of this paper, if a high dose of vitamin B₁ is given the result calculated as the day-dose response (*i.e.* days of cure/dose) will be lower than when a low dose of vitamin B₁ is given. The method of calculating the potency of a substance from days of cure/dose assumes a straight line relationship between duration of cure and dose given. The work in this paper shows that the relationship is curvilinear. Therefore, the day-dose method of calculating results can only be used over a very limited range of duration of cure. It resolves itself into a determination of the dose of the unknown substance required to give a cure of some chosen number of days and leaves no means of interpreting results which are of longer or shorter duration than the period chosen.

(d) *Confirmation of the curve relating duration of cure to dose of vitamin B₁ given.*

In the fifth column of Table XII are given the doses of acid clay corresponding to the mean duration of cure in each test. In the last column is stated in units per g. the potency of each substance as determined from each dose tested. The results from the different doses of any one substance are in good agreement

Table XII. *Vitamin B₁ potency of different substances in terms of the I.S., estimated by duration of cure method.*

Material	Dose	No. of birds cured	Average duration of cure (days)	Equivalent dose of acid clay (g.)	No. of units per g.
Acid clay (original sample)	0.015 g.	4	3.75	0.0435	290
	0.03 g.	11	3.7	0.0425	142
	0.06 g.	16	4.4	0.058	97
	0.12 g.	13	7.7	0.320	267
Acid clay (Standard)	0.03 g.	7	2.1	0.022	73
Dried yeast	0.031 g.	2	4.5	0.0605	195
	0.062 g.	5	1.8	0.020	32
	0.125 g.	15	4.4	0.058	43
	0.25 g.	16	5.9	0.1055	42
Vitamin B ₁ solution (a)	0.1 cc.	4	2.0	0.021	21
	(b)	6	1.8	0.020	10
	(a)	7	2.1	0.022	9
	(b)	9	2.5	0.027	8
Extract of rice polishings	0.25 cc.	5	1.6	0.0185	7
	0.5 cc.	10	5.1	0.0775	15
	1.0 cc.	10	5.7	0.098	10
	2.0 cc.	11	7.5	0.256	13
Yeast (soft extract I)	0.12 g.	3	1.7	0.019	16
	0.24 g.	6	3.5	0.039	16

except for (a) certain doses for which a relatively small number of animals was used, and (b) the highest dose of acid clay of which the result has to be interpreted from a very high, and therefore unreliable, part of the curve. The curve may, therefore, be considered a fairly reliable one for relating duration of cure to dose of vitamin B₁ given.

Moreover this curve and the curve relating percentage of birds to dose of vitamin B₁ give the same results within the limits of accuracy of the tests.

B. METHOD OF CALCULATING RESULTS BY PERCENTAGE OF BIRDS CURED.

(a) *Standard deviation of the percentage number of birds cured.*

The percentage standard error of each of the 19 tests was calculated from the formula $\epsilon = \sqrt{\frac{p \cdot q}{n}}$ where p = the percentage of pigeons cured, q = the percentage not cured, n = the total number of pigeons used in the test. From each value of ϵ the corresponding value of σ was calculated ($\sigma = \epsilon \sqrt{n}$). The weighted mean of the 19 values for σ was found to be 43.08 %.

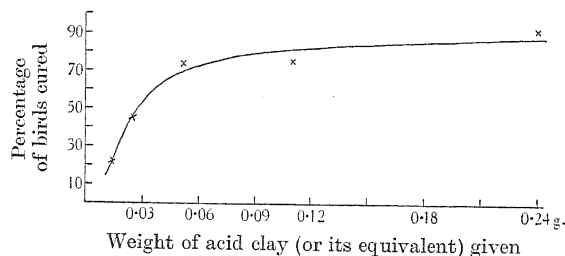


Fig. 8. Curve of response constructed from percentage of polyneuritic birds cured by doses of activated acid clay or the equivalent of these doses in other substances.

(b) *Relation of percentage of birds cured to dose of vitamin B₁ given.*

A curve of response (Fig. 8) has been constructed by grouping the pigeons and averaging results as in the last section. This curve is, therefore, based on more data than that in Fig. 2, it is extended through higher doses and it relates percentage of birds cured directly to the corresponding dose of the standard.

(c) *The degree of accuracy obtainable by this method.*

The degree of accuracy of an estimation by the use of 9 pigeons has been worked out as for the calculation of the duration of cure. It is summarised in Table XIII.

Table XIII. *The probabilities of obtaining certain degrees of accuracy in this test. Standard error of percentage of birds cured when 9 birds are used in a test.*

$$\epsilon = \frac{\sigma}{\sqrt{n}} = \frac{43.08}{\sqrt{9}} = 14.36.$$

Percentage no. of pigeons cured, say,	Abscissa corresponding to % cured	Range of percentage number cured	Range of abscissae* corresponding to range of % number cured	Percentage range of abscissae
50	0.028	(a) $\pm \frac{2}{3} \epsilon = \pm 9.57$ ($\frac{1}{2}$ chance) 59.57 40.43	0.036 0.021	129 77
		(b) $\pm \epsilon = \pm 14.36$ ($\frac{2}{3}$ chance) 64.36 35.64	0.042 0.019	150 67
		(c) $\pm 2 \epsilon = \pm 28.72$ ($\frac{3}{2}$ chance) 78.72 21.28	0.084 0.014	300 50

* Obtained by direct reading from curve of response.

It is evident from the figures in the last column of Table XIII that the curve relating the percentage of birds cured to dose of vitamin B₁ given is somewhat flatter at its upper end than the curve relating the duration of cure to dose of vitamin B₁ given. Except for results falling on the upper part of the curve, which one would try to avoid in either test, it is evident that equal degrees of accuracy are obtained in the estimation of vitamin B₁ by the use of polyneuritic pigeons in either way, provided the results are interpreted by means of curves of response such as we have constructed for this paper.

2. RATS.

THE GROWTH RESPONSE.

(a) *The standard deviation of the response to a dose of vitamin B₁.*

The mean variance of the increase in weight in 3 weeks of rats given a dose of vitamin B₁ after they have ceased to grow on a diet deficient in this vitamin is 89.06 for bucks and 38.96 for does. Thus the standard deviation of the increase in weight in 3 weeks is 9.4 for bucks and 6.2 for does. These figures are presented with some hesitation as they were calculated from only 47 bucks and 36 does respectively. For purposes of calculating the degree of accuracy obtainable in the estimation of vitamin B₁ as carried out by the growth-promoting test in this laboratory, the two values for σ have been averaged ($M = 7.8$).

(b) *The degree of accuracy obtainable by this test.*

If 7.8 be accepted as the value for σ then the standard error of the test using 9 rats on a dose is $\epsilon = \frac{\sigma}{\sqrt{n}} = \frac{7.8}{3} = 2.6$. The degree of accuracy may then be calculated from the equation of the curve of response, $y = 139.4 + 70.4 \log x$ (Table XIV).

Table XIV. *Degree of accuracy obtainable with 9 rats on a dose.*

$$\left[\epsilon = \frac{\sigma}{\sqrt{n}} = \frac{7.8}{3} = 2.6 \right].$$

Average increase in wt. in 3 weeks, say,	Abscissa corresponding to this increase	Range of increase in wt. in 3 weeks (g.)	Range of abscissae corresponding to range in increase in weight	% range of abscissae (doses)
20 g.	0.0201	(a) $\frac{2}{3}\epsilon = \pm 1.74$ ($\frac{1}{2}$ chance) 21.74 18.26	0.0213 0.0190	106.1 94.5
		(b) $\epsilon = \pm 2.6$ ($\frac{2}{3}$ chance) 22.6 17.4	0.0219 0.0186	108.6 92.6
		(c) $2\epsilon = \pm 5.2$ ($\frac{3}{2}$ chance) 25.2 14.8	0.0239 0.0170	118.9 84.5

Similarly the percentage range of abscissae, *i.e.* the percentage range of accuracy obtainable when only 4 rats are used on a dose of vitamin B₁ is:

1 in 2 chance (probable error)	92.6 to 108.6 %
2 in 3 chance	88.2 to 113.8 %
21 in 22 chance	77.6 to 129.5 %

Thus the degree of accuracy obtainable in an estimation of vitamin B₁ appears to be very high. As, however, the value for the standard deviation is based on only a small number of animals, it is possible that the degree of accuracy may not be as great as this, though it is unlikely that it will be very much less.

SUMMARY.

New methods are proposed for estimating the potency of preparations containing vitamin B₁ by means of (1) tests in which pigeons are cured of head retraction, (2) tests in which the growth of rats is observed. Several substances have been examined by these methods in comparison with the International Standard (I.S.).

The responses of pigeons (duration of cure and percentage of birds cured) and of rats (growth) to doses of vitamin B₁ are curvilinear. This fact must produce differences in results calculated by Peters's day-dose method according as the dose tested is high or low. With pigeons, the duration of cure and the percentage of birds cured give the same estimation of potency provided the results are interpreted by means of suitable curves relating response to dose of vitamin B₁ given.

The rat and pigeon methods give the same estimation of potency of a soft extract of yeast in comparison with the I.S. Thus the rat and pigeon appear to have equal ability for dealing with the vitamin B₁ in the Standard. In tests

of two different samples of dried yeast, however, the pigeon test indicated a vitamin B₁ potency about double that indicated by the rat test. Thus the rat appears to be less able to respond to the vitamin B₁ of dried yeast than does the pigeon. Statistical estimations of the results indicated that the probable errors of the tests using 9 animals in a test are:

- | | | | |
|---|-----|-----|-------------------|
| (1) pigeon, (a) duration of cure | ... | ... | + 31 or - 26 %; |
| (b) percentage cured | ... | ... | + 29 or - 23 %; |
| (2) rat, mean increase in weight in 3 weeks | ... | | + 6.1 or - 5.5 %. |

Moreover, the standard deviation of results obtained by pigeons is no greater in tests on solids (*e.g.* acid clay or dried yeast) than it is for liquids (*e.g.* extracts of yeast). Therefore, there is apparently no greater variation in the ability of different birds to deal with solids than in their ability to deal with liquids.

In spite of the probable error of the pigeon test being much greater than that of the rat test, the former has the great advantage of being specific for the factor it is used to estimate. It may also be that it gives a truer result with certain substances (*e.g.* dried yeast) than the rat test, though it may be difficult to use the polyneuritic pigeon for testing many substances which cannot be suspended in water and given by a stomach tube.

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THE BIOCHEMICAL JOURNAL

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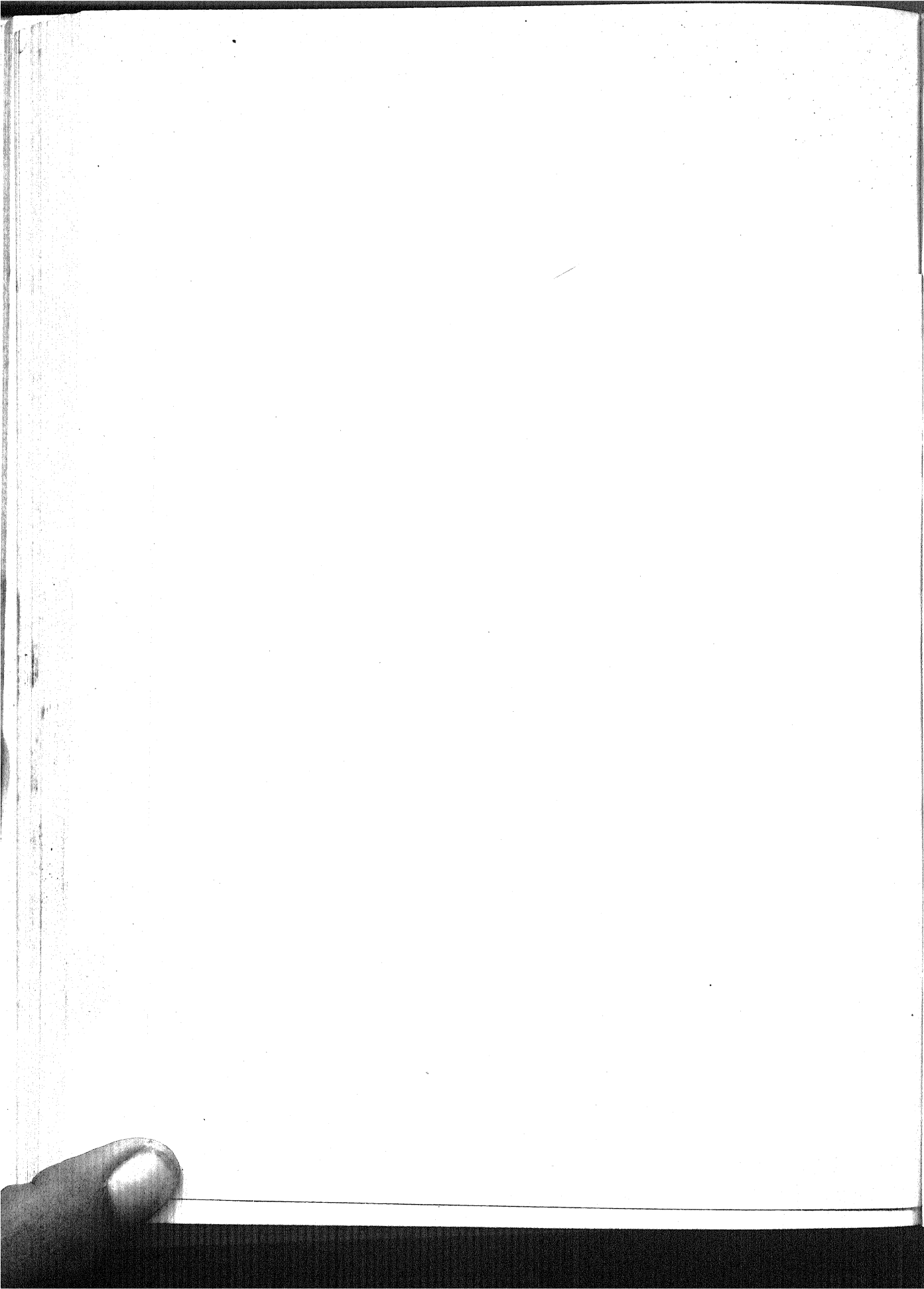
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THE BIOCHEMICAL JOURNAL, 1933

ERRATUM

Vol. XXVII, p. 1753, line 22

for 1 cc. *M*/45 phosphate read 1 cc. *M*/15 phosphate



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CCXXXV. REFRACTOMETRIC EXAMINATION OF PURIFIED ANTITOXINS.

By CHARLES SIEBENMANN.

From the Connaught Laboratories, University of Toronto.

(Received July 15th, 1933.)

PURIFIED diphtheria and tetanus antitoxins, prepared from antitoxic horse sera by isolating the water-soluble globulins, are purer than the original sera in so far as they contain more antitoxin per g. of protein. Their higher degree of purity however does not exclude the fact that for a definite volume they often contain more protein than the original sera from which they were prepared. As the injection of concentrated protein solutions is not desirable, there are regulations fixing the maximum protein or total solid content of such purified antitoxins. For institutions concerned with the production of such antitoxins, the determination of total solids or total protein is therefore a matter of daily routine.

The gravimetric methods for determining total solids and protein, and the Kjeldahl method, are well established but rather lengthy procedures. The nephelometric method for determining proteins [Kober, 1917] is quicker, but it seems not easy to obtain reproducible results. The present paper deals with the refractometric determination of the globulin content of concentrated antitoxins.

The refractometric determination of serum-protein by Reiss [1913; 1915; 1924] differs from the method described here in that in natural sera the concentration of non-protein substances is unknown, and may in exceptional cases show great variations, so that from the refractive index alone the protein concentration can only be determined approximately. However, antitoxins, purified by a modification of Banzhaf's method of salt precipitation and heating, represent pseudoglobulin solutions free from albumin and non-protein fractions and containing known amounts of salt and preservative. The protein content of such solutions can be determined by a single refractometric reading.

Basis of the refractometric globulin determination.

First we have to prove that a linear relationship between changes in the refractive index and changes in the protein concentration, as observed by Reiss [1903] and Robertson [1909] for a number of proteins, exists also for solutions of antitoxic horse globulin.

From a solution of pseudoglobulin containing 1600 units of diphtheria antitoxin per cc., and freed from salts by prolonged dialysis against distilled water, a series of dilutions was prepared so that each dilution contained exactly 0.80 % NaCl and 0.38 % tricesol.

Table I records the globulin content of the undiluted antitoxin, the globulin content of each dilution (*c*) as well as the refractive index (*n*) determined by means of a Zeiss dipping refractometer, at 17.5°.

Table I. *Diphtheria antitoxin 512 C.*

Before dilution 1 cc. contains 1600 antitoxin units and 24.68 % globulin (by weight).
All solutions contain 0.80 % NaCl + 0.38 % tricesol.

Globulin content C* %	Refractive index n	Change of n per 1 % globulin a
6.17	1.34675	0.00185
4.63	1.34391	0.00186
4.11	1.34300	0.00187
3.08	1.34101	0.00185
1.54	1.33810	0.00181
		Average 0.00185

n' = refractive index of NaCl 0.80 % + 0.38 % tricesol = 1.33531.

* Calculated from dilutions.

If for such solutions, the relation between refractive index and globulin concentration is linear, then the following equation must hold true:

$$n = n' + ac \quad \text{.....(1),}$$

where n is the refractive index of the solution, n' a constant, being the refractive index of the solvent (solution of 0.80 % NaCl + 0.38 % tricesol) and c the globulin concentration. The value a represents the supposedly constant change in the refractive index (n) due to the presence of 1 % horse pseudoglobulin.

In Table I, col. 3, are recorded the values of $a = \frac{n - n'}{c}$ calculated from equation (1). These represent the changes in the refractive index caused by the presence of 1 % globulin.

Table II shows the results of a similar series of experiments, in which the solutions examined were prepared from the same antitoxin, but contained no salt or tricesol. Here, too, the values of a show a high degree of constancy and are practically identical with those recorded in Table I. The presence in solution of 0.38 % tricesol and 0.80 % NaCl, seems, consequently, not to change the refractive properties of pseudoglobulin.

Table II. *Diphtheria antitoxin 512 C.*

Before dilution 1 cc. contains 1600 antitoxin units and 24.68 % globulin (by weight).
All dilutions are free from salt and tricesol.

Globulin content C* %	Refractive index n	Change of n per 1 % globulin a
8.23	1.34834	0.00184
6.18	1.34688	0.00187
4.63	1.34389	0.00186
3.71	1.34215	0.00184
1.85	1.33877	0.00185
		Average 0.00185

n' = refractive index of distilled water = 1.33320.

* Calculated from dilutions.

The same values of a were obtained by examining diluted antitoxin solutions prepared from another diphtheria antitoxin containing 1800 units per cc. (Table III).

Table III. *Diphtheria antitoxin 512 A.*

Before dilution 1 cc. contains 1800 antitoxin units and 18.55 % globulin (by weight).
All dilutions contain 0.80 % NaCl + 0.38 % tricresol.

Globulin content C* %	Refractive index <i>n</i>	Change of <i>n</i> per 1 % globulin <i>a</i>
9.27	1.35258	0.00186
6.18	1.34688	0.00187
4.63	1.34389	0.00186
3.71	1.34215	0.00184
1.85	1.33877	0.00185
	Average	0.00186

$$n' = 1.33531.$$

* Calculated from dilutions.

Finally we had to show that antitoxic globulin does not differ from normal globulin with regard to refractive properties. Hurwitz and Meyer [1916] and later Meyer, Hurwitz and Taussig [1918], made refractometric globulin determinations on antitoxic horse sera, using a salt precipitation method outlined by Robertson [1915]. They apparently assumed that the refractive properties of antitoxic globulin are identical with those of normal globulin, without showing experimental proof. From normal horse plasma, pseudoglobulin was separated in exactly the same way as in the preparation of diphtheria antitoxin. From the salt-free dialysed globulin solution dilutions were made and examined by the refractometer. The results, recorded in Table IV, show that the change in

Table IV. *Solutions of normal horse globulin.*

Before dilution 1 cc. contains 14.54 % globulin (by weight).
All dilutions contain 0.80 % NaCl + 0.38 % tricresol.

Globulin content %	Refractive index <i>n</i>	Change of <i>n</i> per 1 % globulin <i>a</i>
3.63	1.34203	0.00185
2.42	1.33981	0.00186
1.81	1.33871	0.00187
0.45	1.33613	0.00181
	Average	0.00185

$$n' = 1.33531.$$

the refractive index of a normal globulin solution due to the addition of 1 % globulin (col. 3) is exactly the same as for solutions containing antitoxic globulin.

This value ($a = 0.00185$) is considerably lower than the corresponding value for ox serum-globulin ($a = 0.00229$) used by Robertson [1915] but is practically identical with $a = 0.00184$ determined by Reiss [1924] for human serum-protein and with $a = 0.00186$ calculated by Adair and Robinson [1930] for horse globulin.

For a purified diphtheria antitoxin containing a known amount of salt and tricresol, the globulin content can consequently be determined by a single refractometric reading. From the refractive index the globulin content is calculated according to equation (1).

Technique.

Using the attachment No. 1 of the Zeiss dipping refractometer, it is possible to determine refractive indices in the range from 1.325 to 1.367. As the refractive indices of purified antitoxins are generally considerably higher, the

concentrated antitoxins must first be diluted (1:4) in order to make the determination possible.

Example: Globulin determination of diphtheria antitoxin 513 A. Exactly 6 cc. of salt solution "A" containing 0.80 % NaCl + 0.38 % tricesol are placed in a test-tube and 2 cc. of antitoxin measured with an Ostwald-Folin pipette (to contain) are added. The pipette used for the antitoxin should be rinsed out several times with the diluted antitoxin solution. This dilution (1:4) gives in the refractometer at 17.5°, a reading of 42.55, corresponding to a refractive index of 1.34371¹.

From the value (n) and the refractive index of the salt solution ($n' = 1.33531$)

$$c = \frac{n - n'}{a} = \frac{1.34371 - 1.33531}{0.00185} = 4.55 \% \text{ globulin.}$$

Taking into account the dilution (1:4) of the antitoxin, the globulin content of the undiluted antitoxin is $4 \times 4.55 \% = 18.20 \%.$

Globulin determination by means of a chart.

Instead of calculating the globulin content in the way described, it is simpler to read the results directly from a chart. Such a chart (Fig. 1) is worked out

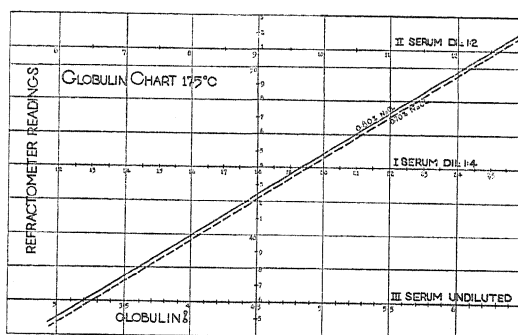


Fig. 1. Ordinates: refractometer scale readings at 17.5°. Abscissae: globulin percentages of the undiluted antitoxin. Abscissae I, for antitoxin examined at dilution 1:4. Abscissae II, for antitoxin examined at dilution 1:2. Abscissae III, for antitoxin examined undiluted.

— Solutions containing 0.80 % NaCl + 0.38 % tricesol.
 - - - Solutions containing 0.70 % NaCl + 0.38 % tricesol.

on the basis of equation (1), using for the constant a the value 0.00185. The globulin percentages are recorded as abscissae, the refractometer readings as ordinates. Using this chart, it is not necessary to convert the refractometer readings into refractive indices. The chart records directly the scale readings of the Zeiss refractometer obtained by examining the antitoxins at the dilution 1:4². These direct readings represent points all lying on a straight line. The fixed points of this chart calculated from equation 1 are tabulated in Table V.

For the determination of globulins with the technique described, it is necessary that the purified antitoxins contain exactly 0.8 % NaCl + 0.38 % tricesol, but the method may be worked out for any definite salt or tricesol concentration by introducing into equation (1) corresponding values of n' . For antitoxins containing e.g. 0.7 % NaCl instead of 0.8 %, n' becomes 1.33519 (20.16) and

¹ Using the conversion table of Reiss [1924].

² Antitoxins with less than 12 % total solids should be examined undiluted or diluted 1:2. Corresponding abscissae are entered in Fig. 1.

Table V.

Fixed points of Fig. 1.

Calculated for antitoxins containing 0.80 % NaCl + 0.38 % tricresol.

Globulin % of undiluted antitoxin	<i>n</i> Calculated for dilution 1:4	Corresponding refractometer reading (17.5°)
12	1.34086	35.00
18	1.34363	42.35
20	1.34456	44.81
24	1.34641	49.71
26	1.34733	52.25

Example. Globulin determination of diphtheria antitoxin 512 A. Dilution 1:4 with salt solution A. Refractometer reading at 17.5° = 42.55 ($n = 1.34371$) indicating on Fig. 1, 18.17 % globulin (against 18.20 % by calculation).

the refractometric readings lie according to equation (1) on a straight line which runs parallel with the 0.8 % line (Fig. 1).

For research work it is often desirable to determine the globulin content of freshly dialysed, salt-free antitoxins. For such cases the same chart can be used, but the dilution (1:4) has to be carried out in the following way. Instead of using saline "A" a special saline "B" containing 1.07 % NaCl and 0.506 % tricresol is needed, bringing the final concentration to 0.8 % NaCl and 0.38 % tricresol. Then the determination can be carried out by means of Fig. 1.

This dilution technique has the advantage over refractometric examination of salt-free globulin solutions in that it applies not only to pseudoglobulin solutions, but also to mixtures of pseudoglobulin and euglobulin, the latter being brought into solution by the saline used for dilution.

Conversion of globulin percentages into total solid figures.

The technique of determining total solids by weight, carried out in connection with this work, is as follows.

In a wide weighing bottle with ground glass stopper, 6 cc. of distilled water are placed. To this exactly 2 cc. of antitoxin are added, using an Ostwald pipette (to contain), the pipette being washed out at least 6 times with the diluted antitoxin. The pipette is finally filled with distilled water and this is added to the diluted antitoxin. The bottle is first heated for 2 hours at 90° and then at 100–105° until its weight becomes constant. By using this technique the total solids can be determined with a maximum error of 0.20 %.

Comparisons of total solid determinations on antitoxins free from tricresol with determinations on the same products, but containing 0.38 % tricresol, show that during the heating all tricresol is volatilised. For converting the globulin content, determined with the refractometer, into total solid figures, we have consequently to add only 0.80 % in weight, taking into account the salt content of the solutions. For salt-free antitoxins, the globulin figures should, within the limits of experimental error, be identical with the total solid figures determined by weight. The mineral content of the tap water, used for dialysis, has no influence on the results, if its total solid content is below 0.1 %, as in our case. This possible cause of error has however to be checked for each place where such determinations are carried out.

Temperature coefficient of the refractometer readings.

As the refractive index of any solution varies greatly with temperature, it is necessary to carry out refractometric measurements at a constant temperature.

In order to be able to carry out our determinations at any given room temperature, the temperature coefficient (K) of the refractometer readings (*i.e.* the change of the scale reading due to a temperature change of 1°) was determined. For the temperature range $17-25^\circ$, K was found to be 0.260 for an antitoxin¹ containing 19.20 % total solids and 0.267 for an antitoxin¹ with 25.38 % total solids, while for distilled water K is lower (0.236). K seems consequently not to be independent of the concentration. For the type of globulin solutions, however, which are in question, the coefficient $K = 0.26$ is sufficiently accurate.

Using this temperature coefficient the globulin contents and total solids of a number of antitoxins were determined by the refractometer at room temperature². The results obtained (Table VI, col. 6) are compared with determina-

Table VI. *Determinations at room temperature.*

Anti-toxin* Seitz-filtered	Temp. °C.	Scale reading	Temp. corrections ($K=0.26$)	Reading calculated for 17.5°	Total solid (Fig. 1)	Total solid† determined at 17.5°	Total solid† by weight
Erysipelas:							
2 A	24.30	40.62	+1.78	=42.40	18.85	18.77	18.79
3 A	24.20	40.97	+1.75	=42.72	19.11	19.10	18.94
Tetanus:							
60 A	24.00	41.87	+1.70	=43.57	19.77	19.77	19.85
B	24.10	41.99	+1.73	=43.72	19.90	19.90	19.79

* Containing 0.80 % NaCl + 0.38 % tricesol.

† Compare Table VII B.

tions done on the same material but carried out at exactly 17.5° (col. 7). The measurements carried out at room temperature yield total solid figures which show satisfactory agreement with the results calculated from the readings obtained at 17.5° and lie very close to the total solid figures determined by weight.

For carrying out the refractometric determinations in the way just described, temperatures above 25° should be avoided and care should be taken that the single determination does not take more than 10 minutes because of possible evaporation of the solutions.

DISCUSSION.

Refractometric globulin determinations (17.5°), using Fig. 1, were carried out on a number of diphtheria, tetanus and scarlet fever antitoxins. The results are shown in Tables VII A and VII B.

The total solid figures in col. 4 are calculated from the refractometric globulin values (col. 3), taking into account the salt content of the antitoxins (see p. 1749). These calculated figures based on the refractometric method show a satisfactory agreement with the total solid figures obtained by weight (col. 5). Of interest is that whatever antitoxin these solutions of horse globulin contain, their refractive properties do not differ from those of normal horse globulin.

The possibility of the turbidity giving rise to error was studied in the case of diphtheria antitoxin (Table VII A). The turbidity (col. 7) of the undiluted bag material, was determined by comparison with turbidity standard suspensions containing definite amounts of fuller's earth (expressed in parts per 1 million parts of the solution). The total solid figures (col. 4) based on the

¹ Containing 0.80 % NaCl + 0.38 % tricesol, dilution 1:4 with saline A.

² Using the water-bath (No. 418111 Zeiss Catalogue on Dipping Refractometer) which was used for all other experiments but without the constant water level (No. 418101 and No. 418133) designed for maintaining the bath temperature at 17.5° .

Table VII A. *Refractometric globulin determination by means of Fig. 1 (17.5°).*

Product*	Refr. reading at 17.5° (dilution 1:4)	Globulin (refr.) %	Total solid (refr.) %	Total solid (by weight) %	Difference†	Turbidity
Diph. ant. Seitz-filtered:						
507,2B	41.43	17.23	18.03	17.83	+0.20	<100
509 A	41.82	17.53	18.33	18.35	-0.02	<100
510 A	42.98	18.52	19.32	19.34	-0.02	—
511 A	42.73	18.30	19.10	19.21	-0.11	<100
512 A	42.93	18.45	19.25	19.08	+0.18	<100
513 A	42.55	18.17	18.97	18.84	+0.13	<100
513 B	42.75	18.30	19.10	18.90	+0.20	<100
514 A	43.41	18.83	19.63	19.52	+0.11	<100
Bag material:						
509	51.55	25.42	26.22	26.41	-0.19	600
515	51.60	25.45	26.25	26.37	-0.12	500
516	50.61	24.58	25.38	25.38	0	500
517	49.55	23.82	24.62	24.83	-0.21	300

* All products examined contain 0.80 % NaCl + 0.38 % tricresol unless otherwise stated.

† Total solids by refractometer *minus* total solids by weight.

Table VII B.

Product*	Refr. reading at 17.5° (dilution 1:4)	Globulin (refr.) %	Total solid (refr.) %	Total solid (by weight) %	Difference
Tetanus ant. Seitz-filtered:					
58 A	42.92	18.47	19.27	19.18	+0.09
58 B	42.74	18.30	19.10	19.31	-0.21
56 A	43.15	18.65	19.45	19.12	+0.33
Repeat	43.00	18.52	19.32	19.29	+0.03
57 A	43.20	18.68	19.48	19.31	+0.17
60 A	43.58	18.97	19.77	19.85	+0.08
60 B	43.75	19.10	19.90	19.79	+0.11
Scarlet fever ant. Seitz-filtered:					
116 A	40.26	16.31	17.11	16.86	+0.25
112 A	42.37	18.02	18.82	19.00	-0.18
Repeat	42.46	18.08	18.88	(19.00)	-0.12
120 A	39.45	15.62	16.42	16.61	-0.19
123 A	40.30	16.35	17.15	16.91	+0.24
121 A	39.75	15.87	16.67	18.81	-0.14
Bag material:					
†124 I	46.67	21.47	21.47	21.37	+0.10
Repeat	46.70	21.53	21.53	(21.37)	+0.16
†124 II	48.52	22.98	22.98	22.96	+0.02
Erysipelas ant. Seitz-filtered:					
2 A	42.30	17.97	18.77	18.79	-0.02
3 A	42.78	18.30	19.10	18.94	+0.16

* All products contain 0.80 % NaCl + 0.38 % tricresol unless otherwise stated.

† Products free from salt and tricresol.

refractometric globulin determinations, are, on the average, slightly lower than the total solid figures determined by weight. However, the differences (max. = -0.21 %) lie within the limits of experimental error encountered with the total solid determinations by weight. The refractometric globulin determinations as such show a high degree of accuracy (see repeats in Table VII B). Using calibrated pipettes for preparing the antitoxin dilutions, the refractometric readings are reproducible within less than 0.2 division of the refractometer scale corresponding to a variation of less than 0.2 % in the globulin figure (see Fig. 1).

SUMMARY.

1. The refractive properties of globulin solutions containing diphtheria antitoxin were investigated. No distinct differences were found between these and the refractive properties of normal horse globulin solutions. These findings were confirmed for purified tetanus, scarlet fever and erysipelas antitoxins.

2. A refractometric method for routine determination of globulins and total solids of purified antitoxins by a single refractometer reading is described. From this refractometer reading the globulin concentration is obtained either by calculation or by using a reference chart.

Instead of carrying out the determinations at 17.5° , the readings can be made at any definite room temperature ($17-25^{\circ}$) taking into account the temperature coefficient of the refractometric readings.

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CCXXXVI. OXIDATION OF FATTY ACIDS IN THE LIVER¹.

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(Received September 4th, 1933.)

SINCE the classical experiments of Embden, Knoop and Dakin over 25 years ago, little work has been carried out on the oxidation of fatty acids in the body. This has been due perhaps to the difficulties inherent in perfusion technique. The advent of the comparatively modern manometric methods of Barcroft and Warburg has made it possible to study fatty acid oxidation, and the various factors influencing it, in isolated organs in a strictly quantitative manner. We propose to describe in this paper the results of experiments carried out with these methods using thin tissue slices, usually not exceeding 20 mg. dry weight.

Methods.

Tissue slices about 0.4 mm. thick were prepared from fresh guinea-pig liver according to Warburg's methods. The slices were bathed in 0.9 % saline for a few minutes before immersion in the vessels of the manometer. Both the Warburg and Barcroft apparatus have been used. The dry weights of the tissue slices varied from 8 to 20 mg.

The medium in which the tissue slices were immersed consisted of 1.5 cc. Locke solution, 1 cc. *M*/45 phosphate buffer solution p_H 7.4 and 0.5 cc. of a solution of the fatty acid neutralised with sodium hydroxide (to p_H 7.4). In control experiments the solution of the fatty acid was replaced by 0.9 % saline.

Air was displaced by oxygen in all experimental vessels as soon as the tissue slices had been added to the media in the manometric vessels. CO_2 was absorbed by rolls of filter-paper, moistened with 6 % KOH, contained in the inner tubes of the vessels.

After attainment of temperature equilibrium readings of O_2 uptake were taken every 15 minutes for 3 hours. At the end of this period the slices were removed from the vessels, washed in distilled water and dried at 105°, after which they were weighed. The solutions in the vessels were investigated either qualitatively by colour tests or quantitatively by methods which will be described later.

In determining Q_{O_2} (i.e. mm.³ of O_2 absorbed per mg. dry weight tissue per hour) the reading over the first 15 minutes was usually neglected, and the average oxygen uptake was calculated over the remaining period of the experiment.

In all experiments described in this paper guinea-pig liver was used. The animals were mostly young (about 11 oz. weight) and well fed (bran, oats and greens). The animal was well bled before the liver was removed. Tissue slices were taken usually within 15 minutes of the death of the animal.

¹ The substance of this note was read before the Third International Congress for Cytology in August, 1932.

Oxidation of fatty acids etc. by the liver.

Representative values of the respiration of guinea-pig liver and of the development of acetone or acetoacetic acid (as indicated by the nitroprusside reaction), in the presence of the first eight members of the fatty acid series, are shown in Table I. The 2nd and 3rd columns of this Table give the Q_{O_2} for an

Table I. *Oxidation of fatty acids etc. by guinea-pig liver. Initial $p_H = 7.4$.*

Substrate	Q_{O_2} 0.017M	Nitroprusside reaction	Q_{O_2} 0.067M	Nitroprusside reaction
None	4.5	Nil	.	.
Formic acid	4.2	Nil	.	.
Acetic acid	7.4	+++	5.5	++
Propionic acid	10.0	Nil	7.0	Nil
Butyric acid	12.0	++++	10.0	++++
n-Valeric acid	12.8	Trace	12.2	Trace
n-Hexoic acid	13.6	++++	5.0	++
n-Heptoic acid	13.8	+	2.8	Nil
n-Octoic acid	13.0	++++	2.8	Nil
Crotonic acid	8.2	++++	8.2	++++
Isocrotonic acid	.	.	7.6	++++
Lactic acid	6.5	?	.	.
Pyruvic acid	5.0	+	.	.
Glucose	4.3	Nil	.	.
Glycerol	5.2	Nil	.	.

initial concentration of 0.017M, and comparative estimates of the intensities of the nitroprusside reaction yielded by the substrates at this concentration; the 4th and 5th columns give the Q_{O_2} for an initial concentration of substrate of 0.067M and comparative estimates of the nitroprusside reactions yielded at this concentration.

The normal Q_{O_2} of guinea-pig liver (*i.e.* in the absence of any added substrate) is given as 4.5. This is an average of 25 observations, the limits of variation being 2.7 and 6.0. The remaining figures quoted in Table I are representative of the results of at least three experiments.

The following facts are of note.

(1) All fatty acids with the exception of formic acid increase markedly the respiration of liver. Acetic acid has the least effect.

(2) There exists an optimum concentration for each fatty acid, above which any increase in concentration leads to a fall in Q_{O_2} and also to the development of acetone bodies, as indicated by the nitroprusside reaction. This is particularly noticeable with higher members of the series. Table II shows the variation of Q_{O_2} with concentration of butyric acid.

Table II. *Oxidation of butyric acid by guinea-pig liver. Variation of Q_{O_2} with concentration of fatty acid.*

Liver alone	Q_{O_2}
+ butyric acid 0.0017 M	4.5
+ " 0.0035 M	6.6
+ " 0.0170 M	11.0
+ " 0.0670 M	12.0
	10.0

The rate of O_2 uptake by liver in the absence of added substrates becomes constant after about the first 30 minutes.

Similarly in the presence of fatty acids at the initial concentration of 0.017 *M* the rate of O_2 uptake is linear. With fatty acids at higher concentrations there is a considerable departure from linearity, this becoming particularly observable with *n*-hexoic and higher fatty acids. In Table III are recorded the rates of

Table III. *Rate of O_2 uptake by guinea-pig liver in presence of fatty acids 0.067 M. Initial $p_H = 7.4$.*

Fatty acid	Q_{O_2} after 30 mins.	Q_{O_2} after 60 mins.	Q_{O_2} after 90 mins.	Q_{O_2} after 120 mins.
Control (no fatty acid added)	6.6	4.7	4.7	4.7
<i>n</i> -Valeric	13.7	13.0	12.2	12.2
<i>n</i> -Hexoic	9.8	6.3	4.4	3.8
<i>n</i> -Heptoic	7.0	3.5	2.5	1.5

respiration of guinea-pig liver, alone and in the presence of *n*-valeric, *n*-hexoic and *n*-heptoic acids, at particular periods during the experiment. Thus with valeric acid the rate of respiration (Q_{O_2}) after 60 minutes was 13.0 and had only fallen to 12.2 after 120 minutes. With *n*-hexoic acid, however, the rate of respiration was 6.3 after 60 minutes and had fallen to 3.8 after 120 minutes.

The existence of an optimum concentration for each fatty acid for maximum respiration, and the departure from the linear rate of respiration of liver in the presence of relatively high concentrations of the higher fatty acids, are probably to be attributed to increased surface adsorption of the fatty acid leading to an interference with the access of oxygen or of an oxygen carrier.

(3) All the fatty acids with an even number of carbon atoms, including acetic acid, give rise to comparatively large quantities of acetone (or acetoacetic acid). Those fatty acids with an odd number of carbon atoms give rise to little or no acetone (or acetoacetic acid). This result is in complete accord with the results of early perfusion experiments.

(4) Both the unsaturated acids, crotonic and isocrotonic acids, are vigorously oxidised with the production of acetone (or acetoacetic acid).

(5) Glucose appears not to be oxidised by liver and not to give any increase in respiration [see also Dickens and Greville, 1933]. Lactic acid definitely increases the Q_{O_2} [see Dickens and Simer, 1930]—so do pyruvic acid and glycerol, but none of these substances leads to so great an increase in respiration as the fatty acids at equivalent concentrations. Of all substances so far investigated the fatty acids appear to be the most vigorously attacked.

Oxidation of butyric acid by the liver.

The question arose as to how far butyric acid suffered oxidation in the liver. This problem was investigated in the following ways.

(a) Determinations were made of the increased CO_2 output by the liver in presence of butyric acid.

(b) Estimates were made of the yield of acetoacetic acid and acetone produced by butyric acid in liver.

1. *CO_2 output by liver in presence of butyric acid.* O_2 uptakes of guinea-pig liver alone and in the presence of butyric acid 0.017 *M* were determined in a bicarbonate-phosphate-Locke medium of the following composition: 1.5 cc. Locke solution, 0.25 cc. phosphate buffer *M*/15 p_H 7.4, 0.47 cc. sodium bicarbonate solution 0.16 *M*, 0.28 cc. saline. To this was added sodium butyrate solution to make a total volume of 3 cc. Air was displaced by an $O_2 + 5\%$ CO_2 mixture and determinations of changes of volume were obtained using a Barcroft

differential manometer. No KOH was placed in the inner tubes of the manometer vessels.

The changes of volume obtained were the differences between O_2 absorbed and CO_2 produced and could be compared with experiments carried out under similar conditions but where provision was made to absorb all CO_2 produced. In such experiments the bicarbonate was replaced by Locke solution, the O_2 - CO_2 mixture by O_2 , and KOH was placed in the inner tubes of the vessels.

In a number of such experiments it was shown that little or no extra CO_2 was produced by the combustion of butyric acid in liver. A typical result is shown in Table IV, the increase in Q_{O_2} due to butyric acid being 5.7 and the

Table IV.

Guinea-pig liver		Q_{O_2}	Q_{CO_2}
" + butyric acid 0.017 M		4.2	3.7
Increase		9.9	4.0
		5.7	0.3
		Q_{O_2}	Q_{CO_2}
		Exp.	
Guinea-pig liver + butyric acid 0.017 M	1	9.4	2.8
	2	9.9	.
	3	13.3	4.2
	4	13.3	.
			4.2
		Increase in Q_{O_2} due to butyric acid	$Q_{\text{acetone bodies}}$ (iodoform method)
		Exp.	$Q_{\text{acetoacetic acid}}$ (aniline method)
		1	2.5
		2	4.2

increase in Q_{CO_2} (mm.³ CO_2 produced per hour per mg. dry weight of tissue) only 0.3.

There is thus reason to believe that although a vigorous oxidation of butyric acid occurs in the liver this does not result in an extensive breakdown to CO_2 . Presumably, therefore, if acetoacetic acid is a product of oxidation of butyric acid in the liver, this does not break down markedly in this organ to acetone and CO_2 . This conclusion can be put to the test by direct analysis.

2. *Estimations of acetone and acetoacetic acid.* Two methods have been employed.

(a) *Iodoform method.* In this method 2.5 cc. of the solution containing the acetone bodies were placed in a large boiling-tube contained in a water-bath and 1 cc. $N H_2SO_4$ was added. Air was led through the solution into a large tube containing 5 cc. 5N NaOH and 5 cc. $N/20$ iodine solution. The water in the bath was brought to the boil, air being drawn slowly through the solution containing the acetone bodies into the alkaline iodine solution. In usually 30–45 minutes all the acetone (together with that contained in the acetoacetic acid) had been taken over into the iodine solution, less than 0.5 cc. liquid remaining in the tube in the water-bath. The alkaline iodine solution, containing an iodoform precipitate, was carefully neutralised (at 0°) and the liberated iodine titrated with $N/20$ sodium thiosulphate solution. This method was found to be accurate only for quantities of acetone corresponding to 1 cc. or less of $N/20$ iodine solution. It does not of course differentiate between any volatile compounds which can combine with iodine in alkaline solution.

(b) *Aniline hydrochloride method.* This method is identical in principle with that recently used by Krebs [1933] and by Postern [1933] in the estimation of oxaloacetic acid. It differs somewhat in detail from that of these workers. The method depends upon the fact that one molecule of acetoacetic acid is broken down in presence of aniline to yield one molecule of CO_2 which can be estimated manometrically. We have adopted the following procedure.

2.5 cc. of the solution containing acetoacetic acid are placed in the left-hand vessel of a Barcroft manometer and to this is added 1 cc. $M/5$ acetate buffer p_{H} 3.8. A small cup, containing 0.1 g. aniline hydrochloride, is hung by means of a platinum hook on the inner tube of the Barcroft vessel. In the right-hand vessel are placed 2.5 cc. saline together with 1 cc. $M/5$ acetate buffer p_{H} 3.8. The manometer is placed in a bath at 37° and allowed to shake at the usual rate for 10 minutes after which the taps are closed and the manometer again allowed to shake. Usually no change in the manometer levels occurs within 10–15 minutes, showing little or no spontaneous breakdown of acetoacetic acid under these conditions. The cup containing the aniline hydrochloride is displaced by a sharp shake of the manometer and in a few minutes there is rapid change in the manometer levels as the aniline reacts with the acetoacetic acid. Readings are taken every 5 minutes. The reaction appears to be complete in 30 minutes but we usually take readings for a further 30 minutes. We have not dealt with quantities of acetoacetic acid appreciably greater than that corresponding to an evolution of 250 mm.³ CO_2 .

This method, which is quick and gives reproducible results, estimates acetoacetic acid and not acetone or acetaldehyde. Were oxaloacetic acid present in small quantity it would be, in all probability, fully decomposed under these experimental conditions before the aniline hydrochloride was added [see Postern, 1933].

The acetone bodies, estimated either by the iodoform method or by the aniline hydrochloride method, are calculated in terms of mm.³ produced per mg. dry weight of tissue per hour, and are thus given in units directly comparable with the Q_{O_2} . Representative results are shown in Table IV. Taking two experiments in which respirations of guinea-pig liver in presence of sodium butyrate $0.017 M$ were identical, $Q_{\text{acetone bodies}}$ (iodoform method) proved to be identical with $Q_{\text{acetoacetic acid}}$ (aniline method). This indicates that no appreciable quantities of acetone or acetaldehyde are formed in the combustion of butyric acid by liver; otherwise the $Q_{\text{acetone bodies}}$ would be greater than $Q_{\text{acetoacetic acid}}$.

If butyric acid is burned completely to acetoacetic acid it would be expected that the $Q_{\text{acetoacetic acid}}$ would be equal to the increase in Q_{O_2} due to the butyric acid. This does not appear to be the case. Two typical results are shown in Table IV where it will be seen that the $Q_{\text{acetoacetic acid}}$ is about half the value of the increase in Q_{O_2} .

It was conceivable that some of the butyric acid was oxidised to succinic acid, which would then be rapidly oxidised by liver to *l*-malic acid. The latter is only very slowly oxidised by liver and it would be anticipated that *l*-malic acid would accumulate and be observable polarimetrically if succinic acid were formed. Polarimetric examinations, however, gave negative results, so that it must be concluded that oxidation of butyric acid to succinic and *l*-malic acids does not take place to any marked extent in the liver.

It is more likely that the extra oxygen taken up, and not accounted for by the acetoacetic acid, is to be found in β -hydroxybutyric acid, which we have not yet estimated. It has been shown by Snapper and Grünbaum [1927, 1, 2] in perfusion experiments that liver has a marked reducing action on acetoacetic acid. These writers have also shown, by perfusion experiments, that the power of surviving liver to break down acetoacetic acid is small, a conclusion with which the results given above are in agreement.

The action of propionic acid on the oxidation of butyric acid in the liver.

It was shown, in Table I, that propionic acid is oxidised by guinea-pig liver but that it does not give rise to acetoacetic acid.

If propionic acid be added to butyric acid, the formation of acetoacetic acid by the latter in presence of liver is markedly reduced, though there is little change in the uptake of oxygen. Typical results are shown in Table V. The

Table V. *Effect of propionic acid on oxidation of butyric acid by liver.*

				Q_{O_2}	$Q_{\text{acetoacetic acid}}$
Liver + butyric acid	0.017 M			13.3	4.2
"	+ propionic acid	0.0017 M		14.3	3.6
"	+	"	0.01 M	15.6	1.8
"	+	"	0.017 M	14.7	0.7

addition of propionic acid to butyric acid at equivalent concentrations (0.017 M) lowers the $Q_{\text{acetoacetic acid}}$ by 84 %. It is very likely that the effect of propionic acid is due to simple competition with butyric acid for the active surfaces involved in fatty acid oxidation. Another explanation, however, may be that propionic acid is converted in the liver into lactic acid or glucose which, according to clinical observation, exert antiketogenic effects.

This conception was put to the test by examining the effects of glucose and lactic acid on the acetoacetic acid production from butyric acid.

Effects of glucose and lactic acid on the oxidation of butyric acid in the liver.

According to current conceptions the production of acetoacetic acid represents an incomplete oxidation of butyric acid; in presence of glucose the oxidation of the fatty acid should be increased and the production of acetoacetic acid decreased. Experiment (see Table VI) shows no effect, within experimental

Table VI. *Effects of antiketogenic substances on oxidation of butyric acid by liver.*

<i>Glucose:</i>				Q_{O_2}	$Q_{\text{acetoacetic acid}}$
Liver + butyric acid	0.017 M			9.9	2.5
"		+ glucose	0.017 M	9.9	2.8
Liver + butyric acid	0.0017 M			6.6	+++
"		+ glucose	0.017 M	6.9	+++
Liver + crotonic acid	0.017 M			7.1	+++
"		+ glucose	0.017 M	6.7	+++
<i>Lactic acid:</i>					
Liver + butyric acid	0.017 M			10.0	1.9
"		+ lactic	0.017 M	10.8	1.7

error, of glucose or of lactic acid at the concentrations used, on Q_{O_2} of liver in the presence of butyric acid or of crotonic acid, or on the acetoacetic acid production from these substances.

The effect of glucose, therefore, as observed clinically, cannot be due to this substance *per se* in the liver. There is certainly no evidence under these physiological conditions that glucose combines with or removes acetoacetic acid; compare also the results of Wigglesworth [1924], who arrived at a similar conclusion.

Effect of glycogen on oxidation of butyric acid in the liver.

On testing glycogen¹, however, a different picture presents itself. Glycogen (see Table VII) brings about a marked decrease in the production of acetoacetic

Table VII. *Effect of glycogen on oxidations of liver.*

	Q_{O_2}	$Q_{\text{acetoacetic acid}}$
Liver + butyric acid 0.017 M	9.9	2.5
" + glycogen (0.5 %)	7.0	1.0
Liver + butyric acid 0.017 M	9.9	2.8
" + glycogen (0.27 %)	7.8	1.8
Liver + crotonic acid 0.017 M	9.5	3.1
" + glycogen (0.25 %)	7.8	1.8
Liver + <i>n</i> -hexoic acid 0.017 M	12.4	3.0
" + glycogen (0.5 %)	7.8	1.6

acid, but, contrary to current ideas, no increased combustion of the fatty acid. There is in fact a definite decrease in the Q_{O_2} in the presence of glycogen. This occurs with crotonic acid as well as with butyric acid. The results would be consistent with the hypothesis that glycogen induces a "sparing" of fat oxidation, in a sense similar to protein sparing, but further experiment will be necessary to establish this. The investigation so far does not support the conception that the presence of carbohydrate stimulates or completes fatty acid oxidation in the liver.

Effects of increased phosphate concentration on the oxidation of butyric acid in the liver.

As stated earlier the oxidation of butyric acid is carried out in a Locke-phosphate buffer solution, the phosphate buffer being $M/45$ (p_H 7.4). On increasing the latter concentration to $M/15$ (p_H 7.4), a marked fall occurs in Q_{O_2} and acetoacetic acid production. Typical results are shown in Table VIII. This

Table VIII. *Effect of increase in phosphate concentration on butyric acid oxidation.*

	Phosphate concentration	Q_{O_2}	$Q_{\text{acetoacetic acid}}$
Liver alone	$M/45$	4.8	0.20
" + butyric acid 0.017 M	"	10.0	2.00
Liver alone	$M/15$	2.0	0.10
" + butyric acid 0.017 M	"	4.3	0.73

result is not due to any marked change in p_H . It seemed possible that the effect of the relatively high concentration of phosphate was due to removal of calcium ions from the medium and to an alteration of the ionic balance as a whole. To test this, phosphate buffer was replaced by sodium glycerophosphate² solution brought to p_H 7.4. Such a solution acts as a very good buffer at this p_H . It was found that both $M/45$ and $M/15$ glycerophosphate Locke media resulted in the production of the normal figures for Q_{O_2} and $Q_{\text{acetoacetic acid}}$ (see Table IX).

¹ The glycogen used was a commercial (B.D.H.) sample.

² A commercial (B.D.H.) sample.

Table IX.

	Conc. of glycerophosphate	Q_{O_2}	$Q_{\text{acetoacetic acid}}$
Liver alone	$M/45$	4.8	0.10
" + butyric acid (0.017 M)	"	10.0	3.61
" alone	$M/15$	5.4	0.10
" + butyric acid (0.017 M)	"	12.3	4.31

Increase of glycerophosphate concentration did not result in a fall of Q_{O_2} or of $Q_{\text{acetoacetic acid}}$; there was evidence rather of a small increase in these values.

Using $M/15$ sodium glycerophosphate (p_H 7.4) as buffer, replacement of Locke solution by saline resulted in a decided fall in Q_{O_2} and acetoacetic acid production (Table X).

Table X.

		Q_{O_2}	Nitroprusside reaction	Final p_H
Liver alone	Locke sol. present	6.0	Trace	7.4
" + butyric acid 0.017 M	"	12.0	+ + + +	7.4
" alone	Saline present	4.7	Trace	7.4
" + butyric acid 0.017 M	"	7.1	+	7.4

These results indicate the importance either of calcium ions, or of a proper balance of ions involving calcium, for the effective oxidation of butyric acid in the liver. The inhibitory effects of increased concentrations of phosphate ions may be due to removal of free calcium ions, though it is also possible that phosphates themselves may play a definite rôle in the mechanisms which regulate fatty acid oxidation.

Comparison of liver, brain and kidney.

The effects of liver, brain and kidney on butyric and crotonic acid oxidation were compared using the tissue slice method. Typical results are shown in Table XI.

Table XI.

	Q_{O_2}	Nitroprusside reaction
Minced liver	2.1	Nil
" + butyric acid 0.017 M	2.7	Nil
" + crotonic acid 0.017 M	2.6	Nil
Intact liver slices	4.5	Nil
" + butyric acid 0.017 M	12.0	+ + + +
" + crotonic acid 0.017 M	8.2	+ + + +
Brain (slices)	3.1	Nil
" + butyric acid 0.017 M	3.0	Nil
" + crotonic acid 0.017 M	2.0	Nil
Kidney (slices)	12.2	Nil
" + butyric acid 0.017 M	21.3	Trace
" + crotonic acid 0.017 M	20.3	Trace

Brain cannot oxidise butyric or crotonic acid; kidney oxidises both these substances but produces little or no acetone or acetoacetic acid (a result in confirmation of the work of Snapper and Grünbaum [1927, 2]). It is easy to show that in contrast to the liver, kidney rapidly breaks down acetoacetic acid but the mechanism of this decomposition has still to be discovered.

Effects of minced liver.

Minced liver has practically no power to oxidise butyric or crotonic acid (Table XI). It was "minced" by squeezing the liver through a sieve of 1 mm. mesh. No obvious damage to the individual cells had occurred. Yet this rupture of the cell organisation as a whole had destroyed the ability of the organ to accomplish one of its most active metabolic processes. In this sense liver offers a distinct contrast to brain. This organ (again in contrast to the liver) oxidises glucose freely, and minced or chopped brain is also very effective [Quastel and Wheatley, 1932]. It is apparent that with fatty acid oxidation in the liver a number of regulating factors are concerned, one at least of which (? a co-enzyme) depends for its stability or effectiveness on the integrity of the cell organisation as a whole.

SUMMARY.

1. A study has been made of the oxidation of fatty acids by guinea-pig liver using the Warburg tissue slice method. The following results have been obtained.

(a) All fatty acids excluding formic acid increase markedly the respiration of liver; acetic acid has the least effect.

(b) An optimum concentration for each fatty acid exists, above which any increase in concentration leads to a fall in Q_{O_2} and the production of acetone bodies. The rate of O_2 uptake is constant except with relatively high concentrations of the higher fatty acids, when the rate of O_2 uptake falls off rapidly.

(c) In agreement with the results of perfusion experiments, all fatty acids with an even number of C atoms (including acetic acid) give rise to acetone (acetoacetic acid) production. Those fatty acids with an odd number of C atoms produce little or no acetone bodies.

(d) The unsaturated acids, crotonic and isocrotonic acids, are vigorously oxidised to give rise to acetoacetic acid.

(e) The fatty acids are more vigorously attacked, for equivalent concentrations, than lactic acid, pyruvic acid or glycerol.

2. A manometric method of estimating acetoacetic acid is described.

3. Acetoacetic acid is not broken down appreciably to acetone and CO_2 in the liver; it is apparently the final oxidation product of butyric acid in this organ.

4. The addition of propionic acid to butyric acid in presence of liver lowers $Q_{\text{acetoacetic acid}}$ (mm.³ acetoacetic acid produced per hour per mg. dry weight of tissue) without affecting markedly the Q_{O_2} (mm.³ O_2 absorbed per hour per mg. dry weight of tissue).

5. Neither glucose nor lactic acid affects the Q_{O_2} or $Q_{\text{acetoacetic acid}}$ observed for butyric acid in presence of liver. There is no evidence that glucose combines with or removes acetoacetic acid under the conditions of these experiments.

6. Glycogen reduces the Q_{O_2} and $Q_{\text{acetoacetic acid}}$ due to butyric acid.

7. Increase of phosphate concentration reduces Q_{O_2} and $Q_{\text{acetoacetic acid}}$, possibly by removing free Ca ions. Phosphate buffer may be effectively replaced by glycerophosphate buffer; increase in concentration of this does not lead to a fall in Q_{O_2} or $Q_{\text{acetoacetic acid}}$. Replacement of Locke solution by saline results in a diminished oxidation of butyric acid by liver.

8. Brain slices do not oxidise butyric or crotonic acid; kidney slices oxidise butyric and crotonic acids but little or no acetone or acetoacetic acid is formed.

9. Minced liver cannot oxidise fatty acids.

Our thanks are due to the Medical Research Council for a grant in aid of equipment. One of us (A. H. M. W.) is indebted to this body for a whole time grant.

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CCXXXVII. THE DETERMINATION OF BROMINE IN BLOOD.

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(Received October 31st, 1933.)

THE suggested significance of blood-bromine concentration for the diagnosis of the psychoses, particularly of the manic-depressive type [Zondek and Bier, 1932; 1933; Sacristàn and Péraila, 1933], has resulted in much research to obtain a reliable method of blood-bromine estimation [Pincussen and Roman, 1929; Pincussen, 1932; Bier and Roman, 1933; Møller, 1932; Behr *et al.*, 1930; Toxopéus and Van Esvelt, 1930]. The method due to Roman, which has been widely used, has been subjected to some technical criticism [Fleischhacker and Scheiderer, 1933; Hahn, 1933; Holtz and Roggenbau, 1933], and while several accurate methods for the micro-estimation of bromide in the presence of excess chloride have been described [Bobtelsky and Rososkaja-Roseinskaja, 1931; Baitschikov, 1931; Hartner, 1933; Bertram, 1933], no simple method for accurate estimation of bromine in blood seems to be available.

The following method has been elaborated as the result of more than 300 blood estimations, and has been found to give reliable and consistent results.

Protein is first removed by precipitation with tungstic acid. An aliquot portion of the filtrate is treated with potassium hydroxide, evaporated to dryness, and heated at 500° for 20 minutes in a nickel crucible. The extract is acidified and the bromide oxidised to bromine by a chromic-sulphuric acid mixture. This is in such concentration that, while chloride remains unoxidised, bromide oxidation is complete [Bobtelsky and Rososkaja-Roseinskaja, 1931; Evans, 1931]. Bromine is removed by aeration and absorbed in starch-iodide solution. The iodine liberated is titrated with *N*/1000 sodium thiosulphate from a microburette [Pincussen and Roman, 1929].

Apparatus.

The aeration apparatus is similar to that of Hartner [1933] and consists of a 100 cc. conical flask fitted with a two-holed rubber stopper. This carries a tube reaching down to the bottom corner of the flask and drawn out slightly at the end. Maximum aeration of the solution in the flask is thus ensured, when the latter is inclined at an angle of 60°, while small particles of solution are not carried over into the exit tube. The generation of carbon dioxide *in situ* by bicarbonate-acid mixtures, as suggested by Hartner, has been found unnecessary.

The rubber stopper also carries a short glass tube bent at right-angles and connected to a small bubbler. The bubbler used by the author consists of a small test-tube (10 cm. × 1 cm.), fitted with a rubber bung carrying an exit tube, and an inlet tube drawn out to a V-neck capillary at the bottom. A distance of not more than 2 mm. separates the bottom of this tube and that of the test-tube.

Reagents required.

Solution No. 1. 10 % sodium tungstate.

Solution No. 2. $2/3$ *N* sulphuric acid.

Solution No. 3. Concentrated sulphuric acid (36*N*).

Solution No. 4. 20 g. of chromic oxide and 40 cc. of concentrated sulphuric acid in 120 cc. of distilled water.

Solution No. 5. 10 % potassium iodide solution.

Solution No. 6. 0.5 % starch solution made according to the directions of Nichols [1929].

Procedure.

A. *Treatment of blood.* Measure 5 cc. of oxalated blood into a 125 cc. conical beaker add 35 cc. of distilled water and allow the mixture to lyse for $\frac{1}{2}$ hour.

Add 5 cc. of 10 % sodium tungstate solution, mix, and then slowly and with shaking add 5 cc. of $2/3$ *N* H_2SO_4 . Stand for 10 minutes.

Transfer the bulk of the chocolate-brown mixture to a centrifuge-tube and spin at 3000 r.p.m. for 15 minutes.

Pour off the supernatant liquid through a cotton-wool filter into a measuring cylinder. Place 35 cc. of the protein-free filtrate in a 100 cc. nickel crucible with 1 cc. of 25 % KOH (bromide-free) and evaporate to dryness on a boiling water-bath (1 hour).

Heat the crucible in an electric oven at 500° for 20 minutes.

Dissolve the white residue in 3 cc. of water and pour into a 100 cc. conical flask. Wash out the crucible with two further 2 cc. portions of distilled water, making the total volume in the flask equal to 7 cc. (± 0.1 cc.). Cool to 18° and then proceed as follows.

B. *Estimation of bromine.* Add slowly down the sides of the flask, with shaking and strong cooling under the tap, 2.5 cc. of conc. H_2SO_4 . At least 10 minutes should be taken for this procedure, and the first two drops, causing evolution of carbon dioxide, must be carefully added in order to prevent losses of hydrogen bromide in the gas evolved.

Now add to the cold solution (18°) 4 cc. of the chromic-sulphuric acid solution. Allow the solution to run all over the sides of the flask, quickly wipe the top of the flask, and then connect up to the aeration apparatus.

This latter should contain 1 cc. of KI solution and 4 drops of starch solution.

Aerate for about 2 hours with a steady stream of air (about 30 cc. per minute). Renew the absorption tube, and continue aeration for a further 3 hours.

Titrate the iodine liberated in these tubes with *N*/1000 thiosulphate.

Remarks concerning technique. (The following remarks concern the details given in the paragraphs above.)

1. *Treatment of blood.* Lithium oxalate is usually free from bromide and causes no interference with the estimation.

If the lysed solution is allowed to stand for long periods, some loss of bromine occurs.

Table I. *Variation of bromine (γ) found with time of lysis.*

Exp.	Time of lysis				
	10 mins.	$\frac{1}{2}$ hour	1 hour	24 hours	6 days
(a)	—	86	—	70	—
(b)	—	150	—	—	94
(c)	284	340	343	—	—

This is in accordance with the findings of Hastings and Van Dyke [1931] and Woodhouse and Pickworth [1932].

Losses occur if sufficient time is not given for the displacement of bromide from protein combination.

Considerable losses are found if the evaporated filtrate is not heated at 500°. All carbonaceous and nitrogenous matter is thus removed. Some representative figures are given below.

Table II. *Variation of bromine (γ) found with and without heating.*

With heating	293	40	265	42	38
Without heating	41	14	94	39	10

The loss of titre found without heating is probably due to a loose combination between the bromine generated during oxidation and an unsaturated substance in blood.

Cyanides are formed by moderate heating and these interfere with the reaction by the formation of bromocyanides [Lang, 1925].

Although, as Bobtelsky [1930] has shown, magnesium and manganese exert a catalytic effect upon the reaction when present in large concentrations, the small amounts present in normal blood cause no catalysis of chloride oxidation with the chromic acid mixture used.

2. *Estimation of bromide.* The starch-iodide solution used for absorption is such that, while no iodine is carried over in the air stream, the blue colour which develops still remains in the presence of 0.4 γ of iodine.

The time taken for the complete removal of bromine from the solution depends on the rate of aeration, but will also be limited by the rate of oxidation, which is slow with small concentrations of bromide. Large amounts may be estimated quite accurately using as short a time as 10 minutes [Hibbard, 1926]. For small amounts of less than 50 γ (1 γ is equivalent to 0.012 cc. of N/1000 thiosulphate), a longer time up to 5 hours is necessary [compare Bobtelsky and Rososkaja-Roseinskaja, 1931]. Some results of the author are given here for comparative purposes.

Table III. *Variation of bromide (γ) found with time of aeration.*

(Figures in cc. N/1000 thiosulphate.)

Titre in 1 hour	14.74	8.52	6.95	3.31	1.17	0.25	0.08
Titre in 5 hours	15.37	9.00	7.10	3.43	1.35	0.38	0.09
Theoretical titre	15.35	9.02	7.15	3.45	1.36	0.38	0.10

Recovery of added bromide.

Some figures for the recovery of potassium bromide added at various stages in the estimation are given below.

Table IV.

Added to whole blood			Added to tungstic acid filtrate			Added after ignition	
Blood value	Added	Recovered	Blood value	Added	Recovered	Added	Recovered
γ	γ	γ	γ	γ	γ	γ	γ
27	20	17	38	50	47	10	9
27	50	50	950	60	57	50	49
186	100	105	25	240	238	70	68
950	100	98	—	—	—	1470	1465
950	60	54	—	—	—	—	—

CRITICAL DISCUSSION.

A. Initial treatment of blood.

The methods for the preliminary treatment of blood for bromide estimation hitherto recommended, have usually involved an ashing process lasting several hours (compare, however, Hastings and Van Dyke [1931]).

Ignition method. The experience of many authors with iodide estimation indicates that some loss occurs with open ashing, even when the temperature is carefully regulated, and the alkali is present in some excess [Orr and Leitch, 1931; Reith, 1930; Allott *et al.*, 1932]. As a result closed combustion has been recommended [Schwaibold and Harder, 1931; Widmann, 1932]. This loss does not appear to take place in bromide estimations [Bier and Roman, 1933], although little systematic work has been published. Pincussen and Roman recommend a temperature of 460–475° in the presence of 2 cc. of 50 % KOH. The blood-extract after ignition has a pale yellow colour which is not removed completely either by charcoal [Bier and Roman, 1933], or barium sulphate [Turner, 1930], and is probably to be attributed to small particles of carbon in a colloidal or semicolloidal state. Prolonged ignition moreover does not remove the colour but causes loss of bromide. Using this method, the author has found that added bromide is not quantitatively recovered from blood when the former is estimated by the chromic acid method described above.

Table V. *Recovery of bromide (γ) from ignited blood-extracts.*

Bromide in blood alone	76	43	198	125
Bromide in blood + 50 γ Br ⁻	110	92	242	170
Recovery of bromide	34	49	44	45

The effect is not due to a reduction in the oxidation potential of the chromic acid mixture by the blood-salts present, nor was any considerable loss observed by using nickel crucibles instead of platinum [compare Behr *et al.*, 1930; Turner, 1930].

Table VI. *Effect of type of ignition vessel (mg. Br⁻ per 100 cc.).*

Platinum	0.54	0.97	2.00	0.98	0.26	5.16	0.79
Nickel	0.52	0.93	1.83	0.98	0.27	4.82	0.77

The losses may possibly be due to the adsorption of bromine ions from the strongly acid oxidation mixture by carbon particles [compare Tezler, 1926].

The relatively low values for blood-bromine usually obtained by the ignition method become more apparent when compared with those obtained by the precipitation technique described below.

Table VII. *Comparison of ignition and precipitation methods for blood-bromide (mg. per 100 cc.).*

Ignition in platinum vessel	0.52	0.14	1.85	0.98	12.10
Precipitation with tungstic acid	0.60	0.18	2.13	0.92	12.72

Precipitation method. The preliminary treatment of blood by the Folin-Wu method (precipitation with tungstic acid) involves certain approximations. It is assumed that the bromine present distributes itself equally between the protein precipitate and the aqueous filtrate. The observation of Pincussen [1929] that 1/3 of the bromine in normal blood is in the state of a non-diffusible organic compound [compare also Guillaumin and Merejkowsky, 1933], suggests that relatively large quantities of bromine may be present preferentially in the protein precipitate, as found with blood-iodine. All the evidence, however, indicates that any organic bromine compound, if present, is readily decomposed either by mild acid or alkali.

Large concentrations of bromide appear to be distributed equally between precipitate and filtrate, and Hastings and Van Dyke [1931], using a modified

Folin-Wu technique, have been able to recover bromide quantitatively (to within 0.02 mg. Br) from serum and cells on this assumption.

The author has investigated the distribution of the much smaller amounts of bromine present in normal blood and finds a similar equality of distribution. The bromine content found by taking a known volume of filtrate is in close agreement with that found after washing the protein precipitate with successive lots of 50 cc. of water.

Table VIII. *Bromide (γ) recovered by washing the precipitate.*

Br found in blood-filtrate (vol. 75/100 cc.)	Br found in first washing of ppt.	Br found in second washing of ppt.	Total Br found	Total Br calc. from that found in blood-filtrate
56	14	4	74	75
47	9	—	56	63
45	9	3	58	60
80	15	—	95	107
183	60	10	253	257

Estimation of bromine in the precipitate by the ignition method gives similar results.

1. Bromine found in blood-filtrate	393 γ
2. Total bromine found by ignition	493 γ
3. Total bromine calc. from (1)	524 γ
4. Bromine found in precipitate (ignition)	118 γ
5. Bromine in ppt. calc. from (1)	131 γ

It is clear that the water-extractable bromine is distributed fairly evenly between the precipitate and filtrate and this is perhaps to be expected in view of the almost complete dissociation of protein salts in dilute solutions [Lewis, 1931], and the large effect of neutral salts in reducing any tendency for the establishment of a Donnan equilibrium [Bolam, 1932].

Treatment of the blood-filtrate with silver nitrate and removal of the silver halides by centrifuging has indicated that, even when the blood-bromide is as high as 78 mg. per 100 cc., only extremely small amounts of bromine remain in the solution (less than 0.1 mg./100 cc.), which may perhaps be accounted for in the method, especially in view of the strong catalytic effect of small concentrations of silver upon the chromic acid oxidation of hydrochloric acid [Bobtelsky, 1930; compare Møller, 1932].

B. *Estimation of bromide.*

A method for the estimation of bromide solutions by a single oxidation must not be sensitive to variations of the concentrations of the other salts present. It must allow for the catalytic effect of metallic ions, if any, while it must have a sufficient H ion reserve to make it insensitive to changes of H ion of the same order as that of the bromide estimated. The only available method of this character appears to be that of Hartner [1933], and this the author has not found to be satisfactory. The chromic acid mixture described above is to be preferred since it allows a greater range of bromide estimation in virtue of its larger concentration of hydrogen ions and of chromic acid, while at the same time it is stable to atmospheric oxidation, does not decompose on keeping and is not catalysed by the small amounts of iron, manganese and magnesium present in blood. Tungstic acid and nickel salts do not interfere. The theoretical

limit of accuracy of the method is 0.5γ Br' in the presence of 100 mg. of Cl', this, in practice, being found to be 2γ Br' [compare Bobtelsky and Rososkaja-Roseinskaja, 1931].

The conditions for the accurate estimation of bromide in the presence of chloride using chromic-sulphuric acid mixture here found are in close agreement with those given by Bobtelsky and Rososkaja-Roseinskaja [cf. also, Evans, 1931]. $9.0\text{ }N$ sulphuric acid is recommended, while 3.5% CrO_3 are also present (these authors give 5% CrO_3). The concentration of chromic acid has little effect upon the oxidation potential of the mixture at sulphuric acid concentrations above $4N$.

Results of the estimation of synthetic mixtures of various salts are given below. It has been found that chemically pure potassium chloride is difficult to obtain, the chloride used having a bromide content of 0.014% [cf. Bobtelsky, 1930; Hahn, 1933]. Some estimations on chloride samples are given in Table X.

Table IX. *Effect of various ions on bromide estimation.*

Concentration of ions per 100 cc.			"Bromide" found (γ) using H_2SO_4 conc. of				
Br γ	Cl mg.	Other ions	$5.5N$	$8.3N$	$9.0N$	$10.5N$	$14.0N$
100	—	3.5 g. CrO_3	78	97	99	99	—
60	—	Do.	—	—	58	61	—
50	—	Do.	—	45	48	50	—
10	—	Do.	—	—	9	—	—
100	50	Do.	58	—	105	118	183
50	50	Do.	—	51	58	—	—
0	50	Do.	—	—	7	22	130
100	50	Do. + $N/10$ Na_2SO_4	60	—	104	112	120
50	50	Do. + 5 mg. MgSO_4	—	—	52	—	—
100	50	Do. + $N/10$ tungstic acid	—	—	107	—	—
100	50	1.5 g. CrO_3	63	—	90	102	153

Table X. *Estimation of bromide (γ) in potassium chloride.*

Wt. KCl taken mg.	Specimen used		
	B.D.H. A.R. (untreated)	Crystallised twice	Crystallised 5 times
10	3	—	—
50	28	15	7
100	53	—	—

Experiments are proceeding to determine the significance of blood-bromine in the psychoses.

SUMMARY.

1. A method for the micro-determination of bromine in 5 cc. of blood is described and critically discussed.
2. The method is accurate to within 2γ for amounts of $5\text{--}1000\gamma$ of bromine.

The author wishes to express his indebtedness to Dr J. H. Quastel, for his advice and criticism and to his medical colleagues for their willing co-operation in the collection of blood samples. His thanks are also due to the Medical Research Council for a grant.

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CCXXXVIII. THE COLORIMETRIC DETERMINATION OF VITAMIN A BY THE ALKALI DIGESTION METHOD.

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(Received September 5th, 1933.)

THE alkali digestion method for the colorimetric estimation of vitamin A which was first adopted by Rosenheim and Webster [1927], is used in this laboratory both in work upon tissues from experimental animals and upon samples of human liver obtained at autopsy [Moore, 1932]. Since the latter work has involved large numbers of determinations on specimens available at varying times after death it appeared desirable (1) to study the technique of the method with a view to simplifying the procedure as far as possible so as to permit rapid working, (2) to examine the effect of ageing on the stability of the vitamin in case unsuspected deterioration should be taking place. The present paper is concerned with the collection of information on these two points.

EXPERIMENTAL.

Standard procedure for determination of vitamin A in liver samples.

In early experiments Moore [1930] extracted the vitamin A from alkaline digests of rat liver by four extractions with ether and washed the combined ether extracts with three changes of water. This procedure presumably ensured almost complete extraction of the vitamin and was therefore suitable for application in a limited number of experiments when the reliability of the results was of primary importance. In routine work however the large number of extractions and washings with indefinite volumes of water involves loss of time, both directly and through the increased danger of the formation of emulsions. Steps were therefore taken to simplify the technique so as to obtain results of reasonable accuracy in rapid routine work. It was found that if the digest was given a preliminary shaking with a small proportion of alcohol a single ether extraction was sufficient to extract almost all (usually 90 % or more) of the vitamin A. Moreover, two washings of the ether extract were sufficient to secure freedom from alkali, while by adherence to the correct proportion of alcohol, ether and water the formation of emulsions could be avoided except in rare instances. By the use of the following method it is possible to average 3-4 assays per hour.

Extraction of vitamin. About 5 g. liver are weighed to the nearest 0.1 g. in a 50 cc. beaker, and minced with scissors into 10 cc. of 5 % aqueous KOH. (In the case of specimens of human liver collected at hospitals a convenient plan is to place the specimen in a small bottle containing the potash. Mincing may be postponed until the specimen is received in the laboratory.) The material is then transferred to a 50 cc. conical flask, which is corked and placed

in cold storage if the determination is not to be carried out at once. To digest the tissues the mixture is heated, preferably in a steam-oven, until complete solution has taken place. The digest is then transferred to a 100 cc. graduated funnel and is shaken strongly (for about 10 secs. each time) first with 5 cc. of ethyl alcohol and then with the addition of 50 cc. of ether. (B.D.H. "A.R." ether and ether of sp. gr. 0.730 give identical results in ordinary work.) The layers are allowed to settle out, and the aqueous layer is discarded. 5 cc. of water are now added and vigorously shaken together with the ethereal layer. After settling, the aqueous layer is discarded and the washing completed by gentle agitation with 50 cc. of water. The ether fraction is next filtered by suction through a layer of anhydrous sodium sulphate¹, contained in a sintered glass funnel, into a 100 cc. wide-necked flat-bottomed flask (as used in Soxhlet extractions) the sulphate being washed with a little ether. The ether is evaporated off rapidly on a water-bath, preferably under suction from a filter-pump.

Colorimetric assay. The residue after evaporation, which should be quite clean and dry in appearance², is dissolved in 5 cc. of chloroform (a further dilution is sometimes necessary in the case of specimens giving high values). Portions of this solution, varying from 0.02 to 0.5 cc., according to the amount of vitamin present, are delivered by an appropriate blood pipette into a 1 cm. cell, or a test-tube of 1 cm. bore which has been checked against a cell. The volume is made up to 0.5 cc. with chloroform, and the pipette is washed out by sucking up the chloroform and expelling it again. 2 cc. of the SbCl_3 reagent are then added and the blue colour, which should give a reading of 3 to 5, is matched quickly in a Lovibond tintometer. Results are calculated in Moore's units as follows.

Example. 4.9 g. of liver were digested and extracted. Residue from extract was dissolved in 5 cc. CHCl_3 . 0.02 cc. of this solution made up to 0.5 cc. with CHCl_3 gave a reading of 4 B 1 Y (neglected).

Blue units (B.U.) per g. of liver = $\frac{4 \times 2.5 \times 5}{0.02 \times 4.9} = 500$ to nearest significant figure.

The 2.5 in the top line of the calculation refers to the total volume of the reaction mixture.

The effect of various modifications in the technique of extraction on the apparent vitamin A content.

The following experiments were carried out on various specimens of ox-, pig- and sheep-liver with a view to testing the efficiency of the above technique and examining the effect of variations in procedure.

Technique of digestion. Portions of ox-liver (specimen A) were digested for varying periods (10–90 minutes) with 5 %, or in one case 10 %, aqueous KOH. The digests were then extracted and the residues assayed in the usual way. Although the technique of digestion affected the yield of fat (*i.e.* the degree of saponification) and consequently the blue value per unit of fat, there was no variation in the blue value per g. of liver.

¹ The anhydrous sodium sulphate used in this work was supplied by Messrs B.D.H. and had given good results over a number of years. Just after sending this paper to press, however, the quality of sulphate supplied was changed. The new sulphate, which was a much finer powder, was quite valueless when used in the way described in the text. Dr F. H. Carr kindly submitted alternative types of sulphate for trial. The quality described as "dense" gave satisfactory results.

² The presence of moisture will cause clouding on adding the SbCl_3 reagent. This clouding may be prevented by adding a drop of acetic anhydride before the reagent, but resort to this procedure should be unnecessary if the extraction is carried out correctly.

The action of alcohol in facilitating the extraction of the vitamin. The purpose of using alcohol is both to prevent the formation of emulsions and to facilitate the extraction of the vitamin. The ability of ether, unaided by alcohol, to extract the vitamin from the alkaline liver digests varied from specimen to specimen. The examples given below will serve to illustrate this point.

Example 1. Good extraction of vitamin A by ether alone. 5 g. of ox-liver (B) were digested by heating. Extraction with 50 cc. of ether alone yielded 1000 B.U. per g. of liver. A second extraction with 50 cc. of ether and 5 cc. of alcohol yielded 62.5 B.U. per g. Most of the vitamin A in this case, therefore, could be obtained by one extraction with ether in the absence of alcohol.

Example 2. Poor extraction of vitamin A by ether alone. 5 g. of pig-liver (C) were digested by heating and were extracted with 50 cc. of ether, yielding 5 B.U. of vitamin A per g. A second extraction with 50 cc. of ether and 5 cc. of alcohol yielded 20 B.U. per g. Conversely when 5 g. of liver were first extracted with 50 cc. of ether and 5 cc. of alcohol 20 B.U. per g. were obtained, while a second extraction with 50 cc. of ether and 5 cc. of alcohol gave only 7.5 B.U. per g. In this case therefore ether by itself extracted only a small proportion of the vitamin, but by using ether and alcohol most of the vitamin could be obtained in the first extraction.

Similar results were obtained in the case of a sample of pig-liver (D) richer in the vitamin, which gave a result of 500 B.U. per g. when the extraction was carried out by gentle shaking (5 secs.) with ether in the presence of alcohol, but only 100 (gentle shaking for 4 secs.) to 200 B.U. per g. (vigorous shaking for 30 secs.) in the absence of alcohol. These results afford a good illustration of the action of alcohol in facilitating the extraction of the vitamin.

The technique of digestion also appeared sometimes to affect the ease of extraction by unaided ether. Thus in one series of experiments on pig-liver (E) the surprising result was obtained that ether could extract the vitamin after digestion of the tissues at room temperature, but not if the digestion had been carried out by the usual procedure of heating in a steam-oven. The disadvantages of using unheated digests in routine work lie in the danger of forming emulsions, and loss of time through blockages in the separating funnel.

Efficiency of the standard process of extraction. The following examples will serve to indicate the proportion of the vitamin extracted.

	1st extraction (alcohol and ether)	2nd extraction B.U.	3rd extraction
Sheep-liver (F)	1250	25	—
Ox (G)	350	30	0
Human (H)	25	0	—
Pig (C)	20	7.5	—

In all cases the bulk of the vitamin was obtained in the first extraction.

Comparison of the alkali digestion method with the Soxhlet extraction method using various solvents. A specimen of fresh ox-liver (I) giving 55 B.U. per g. by the alkali digestion method was weighed and minced finely. Portions of 5 g. each were taken, mixed with an equal volume of anhydrous sodium sulphate and extracted for 1 hour in a Soxhlet extraction apparatus with the following solvents—ether, light petroleum, chloroform, acetone, benzene. Although the amount of material extracted seemed to depend largely on the solvent used, the yield of vitamin A remained constant. Thus, in confirmation of the work of Simmonet *et al.* [1931] and of Wolff [1932] good agreement was observed between results obtained by the Soxhlet and alkali digestion methods.

Rate of deterioration of vitamin A in untreated liver and liver digests on standing.

A large specimen of ox-liver (J), rich in vitamin A, was minced, stirred up and divided into 5 g. portions, which were divided into 3 groups and treated as follows: Group (1) immediate digestion by heating with 5 % KOH: Group (2) slow digestion by KOH at room temperature. Group (3) storage at room temperature without any preservative treatment. Portions from each group were taken at intervals and the vitamin A content assayed by the standard procedure. The results of this experiment, and also those of a similar experiment using a pig-liver (K) of low vitamin A content, are shown in Table I.

Table I. *Rate of deterioration.*

Time in days	Group 1 Digested by heating in 5 % KOH	Group 2 Digested by standing at room temperature	Group 3 Standing without KOH at room temperature
Ox-liver (high vitamin A reserves).			
0	1000	1000	1000
3	1000	1000	1000
7	1000	1000	600
14	1000	1000	400
42	600	600	400
Pig-liver (low vitamin A reserves).			
0	20	20	20
7	20	20	10
14	20	17.5	8
21	15	10	4
28	10	8	1
35	3	10	0
42	3	12.5	0

It will be seen that some deterioration took place in all groups after prolonged standing. Deterioration, however, was less rapid in the liver treated with alkali than in the untreated liver. Thus in the untreated liver definite signs of deterioration were evident in 7 days, while no deterioration (except for a very small fall in the case of the pig-liver, group 2) took place in 14 days in the liver treated with alkali. In the ox-liver treated with alkali deterioration to the extent of 40 % was observed after 6 weeks. In the pig-liver deterioration was more rapid.

DISCUSSION.

The procedure for the estimation of vitamin A in tissues described above is designed to permit rapid routine work upon specimens showing very wide variations in vitamin A content, as found in human livers, and is not intended for use when the highest attainable accuracy in individual experiments is required. For work of this type it is recommended that the alkaline digest should be extracted twice or three times with ether, and that the fat obtained should be saponified before carrying out the colorimetric assay. The effect of these refinements on the final result would vary slightly from specimen to specimen. Additional extractions might be expected to increase the final result by as much as 10 %. Saponification should have little effect in the case of normal mammalian specimens, but might cause increased results in the case of livers of abnormally high fat content.

Similarly the experiments on the determination of the vitamin have not been carried out with a view of ascertaining the ideal conditions for ensuring

its stability, but rather to obtain information as to its behaviour under routine conditions involving periods of storage at ordinary temperatures, *e.g.* during transmission by post. Under these conditions the vitamin is more stable in alkali than in untreated specimens, and the tissues should therefore be placed in potash immediately on dissection for this reason as well as for the further consideration of the removal of danger to the worker in the case of pathological specimens. The results indicate that there is little danger of deterioration in digested specimens provided the assay is carried out within 14 days after death.

SUMMARY.

A simplified form of the alkali digestion process for the assay of vitamin A in tissues by the colorimetric method has been devised. The process permits rapid working and gives results substantially in agreement with those obtained by the Soxhlet extraction method.

At room temperature vitamin A deteriorates less rapidly in liver specimens treated with potash than in untreated tissues kept without preservative treatment. In the case of specimens of liver transferred immediately *post mortem* to potash solution and then stored at room temperature no serious decrease in vitamin A content is to be anticipated if the assay is carried out within 14 days after death.

My thanks are due to Dr T. Moore for suggesting this work, and to Dr L. J. Harris for his valuable criticism. I am also indebted to Prof. Myra Sampson for access to certain results obtained during her visit to this laboratory.

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CCXXXIX. STANDARDISED COLLODION MEMBRANES IN LOW PRESSURE ULTRAFILTRATION.

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(Received September 18th, 1933.)

THE value of ultrafiltration as a research weapon is indicated by its increasing use in biological science for separating colloid-free filtrates from colloidal solutions. The modern tendency, following Bechhold [1907], is towards the use of metallic apparatus embodying flat membranes through which filtration is effected under high pressure. For many purposes such technique is invaluable provided due attention is paid to the fundamental principles underlying ultrafiltration [see Augsberger, 1925; Elford, 1933]. However, when ultrafiltrates are required for quantitative analysis, ultrafiltration through collodion tubes at rather low pressures (about 100 mm. Hg) is often to be preferred for the following reasons.

It is not to be expected that ultrafiltration of biological fluids under several atmospheres pressure will yield filtrates typical of living processes—as witness the significantly higher values for ultrafiltrable calcium obtained by Nicholas [1932] who ultrafiltered blood-serum under 10 atmospheres pressure, compared with those reported by numerous investigators using much lower pressures.

An unavoidable defect of high pressure ultrafilters is that the solution being filtered must come into contact with metallic surfaces and usually with rubber-fibre gaskets. Further, metallic ultrafilters are costly to construct.

The technique to be described was primarily devised for measuring ultrafiltrable blood-serum-calcium though it may be adapted to other similar purposes. For the reasons indicated above it involves filtration under 120 mm. Hg pressure through collodion tubes of standard permeability.

EXPERIMENTAL.

Ultrafiltration apparatus. One unit of the all-glass apparatus is illustrated in Fig. 1. The filtration head carrying a collodion tube of about 10.5 cc. capacity fits into the receiver by means of a standard ground joint. The collodion tubes are made of such a diameter that they will slide nicely over the widened end of the tube A. They can be made closely to adhere to the latter by carefully heating round the groove over a micro-flame. A binding of thin twine gives added security to the joint.

Filtration under excess positive pressure (120 mm. Hg) is preferred to the use of suction as recommended by Greenberg and Gunther [1930] as the risk of concentration of the filtrate by evaporation is thereby minimised. For this purpose nitrogen at a known constant pressure is supplied to tube A (Fig. 1) by means of the apparatus described by Folley and Peskett [1933]. As a further precaution against evaporation of the filtrate the side-tube B is connected to a wash-bottle containing a liquid approximately isotonic with the ultrafiltrate.

A battery of six identical units connected in parallel to the pressure supply and wash-bottle is in use at the present time.

Procedure. A collodion tube taken from store under ice-cold water is tied to a filtration-head and the last traces of water removed from its interior by means of a blunt-tipped pipette introduced through A. These operations must be done as rapidly as possible so that the membrane does not dry. The latter is thrice washed out with 3 cc. portions of the fluid to be filtered the last traces remaining from the third washing being removed with the special pipette as before. Violent shaking during washing is avoided so as to minimise frothing. Then 10 cc. of the solution to be filtered are measured into the membrane, the outside of the latter dried with filter-paper and gas pressure applied to A. After 10 minutes the liquid which appears on the outside of the membrane (mainly water expressed from its pores) is removed by filter-paper and collection of the filtrate in a dry receiver begun.

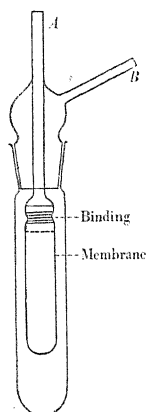


Fig. 1. All-glass ultra-filtration apparatus.

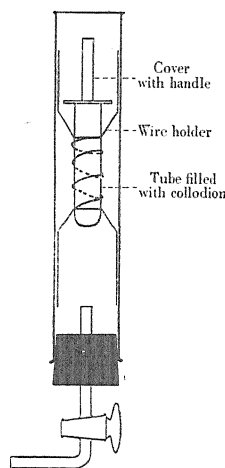


Fig. 2.

In determinations of ultrafiltrable calcium in blood-serum, filtration is allowed to proceed for $2\frac{1}{2}$ hours by which time, with the grade of membrane used, about 2.3 cc. of filtrate are obtained. For analysis 2 cc. are required, the remainder being used to test for absence of protein. Analyses of ultrafiltrates obtained by careful use of this technique show good agreement between duplicates, as the following results indicate. The numbers of cc. of $N/200$ $KMnO_4$ required to oxidise the oxalate, obtained by the Clark and Collip [1925] method from 2 cc. of each of four ultrafiltrates of a sample of bovine blood-serum, were 1.20, 1.21, 1.21 and 1.09 respectively. The titres for three ultrafiltrates of another bovine blood-serum were 1.26, 1.16 and 1.26 cc.

Preparation of collodion tubes. Factors which influence the permeability of collodion membranes include the nature and concentration of the collodion solution, its water content and the time for which evaporation of solvents from the hardening film is allowed to proceed. In quantitative work the importance of using membranes with standard properties needs no emphasis and since the usual technique of making collodion membranes allows little control of the aforementioned factors the following standard method of preparation was devised. In some respects it is similar to a method described by Pierce [1927].

A clean and dry test-tube ground flat at the top is filled with collodion and at once covered by a glass square. When the solution is free from bubbles the tube is inserted into the glass evaporation chamber shown in Fig. 2. It is held in the position shown in Fig. 2 by means of a suitable holder made from wire. The tube is uncovered and the evaporation chamber closed by inserting a rubber bung carrying a metal funnel closed at the bottom by a rubber sleeve and spring-clip. The membrane is cast by rapidly inverting the apparatus and rotating slowly in the hands at a suitable angle for precisely one minute, after which it is clamped in the inverted position, the collodion which has collected in the funnel run off and a metal tube pushed through the stem of the funnel into the position shown in Fig. 3. It is important that the funnel and tube be made of metal since glass

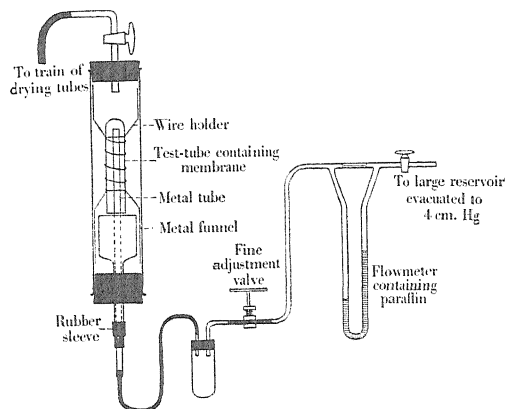


Fig. 3.

components frequently break. The rubber connections shown in Fig. 3 are rapidly made, and at the end of 2 minutes from the start of the casting the taps are opened so that a stream of dry air is drawn through the apparatus and flows over the collodion film. The air is dried by passage through a train consisting of two sulphuric acid wash-bottles, two soda-lime tubes and two calcium chloride tubes. The efficiency of the drying train was tested by placing a weighing bottle containing sulphuric acid in the evaporation chamber, while the air stream was passed for two hours. The sulphuric acid did not appreciably increase in weight during this time.

Variations in the rate of flow are eliminated by constant manipulation of the fine-adjustment valve (Fig. 3), which consists of a large screw-clip with a long handle and fitted with springs to prevent backlash, in such a way that the flow-meter reading remains steady. After an accurately measured period of time the air-flow is cut off, the tube containing the membrane immersed in distilled water, the membrane extracted from the tube and washed in running distilled water for some 24 hours.

Collodion tubes of varying permeability may be made by utilising different evaporation periods or air-flow velocities. For the purpose mentioned above these values are so chosen that the membranes are quite protein-tight and strong enough to withstand the ultrafiltration pressure while giving a satisfactory filtration rate. The membranes referred to in an earlier section are prepared by evaporating at 22° for 15 minutes with an air flow of 13 litres per hour.

The ether-alcohol nitrocellulose solution "Necol" supplied in $\frac{1}{2}$ lb. cans by Nobel Industries Ltd. has proved satisfactory for making both protein-tight and protein-passing membranes. The dilution used is given below, the whole contents

of each can being diluted immediately it is opened. In making membranes the excess collodion must not be used again.

Necol	40 g.
Dry ether	12 cc.
Dry absolute alcohol	8 cc.

Membrane permeability. Information as to the reproducible nature of the membranes can be obtained by comparison of the rates of flow of water through a given area under given conditions of temperature and pressure. These measurements are made by means of the apparatus shown in Fig. 4, the necessary flat

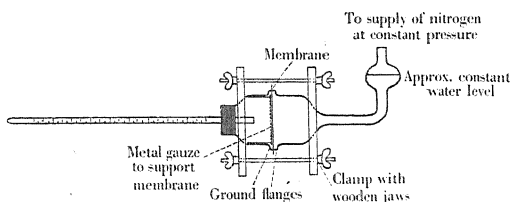


Fig. 4. Jena glass apparatus for membrane permeability measurements.

membranes being obtained by opening up collodion tubes with scissors. The apparatus when assembled is connected to the constant pressure nitrogen supply and the flow of water along the 1 cc. pipette timed with a stop watch.

It is of interest to note here that if these measurements are made on membranes unsupported by metal gauze, the velocity of water flow shows a gradual decrease with time, finally becoming constant. This is partly due to a slow reversible stretching of the membrane since when the latter is mechanically supported the phenomenon is not so pronounced.

The following rates of flow were observed for membranes selected at random from two batches made on different days:

Temperature 21°. Pressure 50 mm. Hg.
Constant membrane area (approx. 6.2 cm.²).
Flow 0.46, 0.50, 0.41, 0.46, 0.43, 0.41 mm.³ per sec.

Keeping in mind the conditions under which these measurements were made, these results show that membranes of reproducible permeability can be made by the method described.

SUMMARY.

A technique has been evolved whereby collodion tubes of standard and reproducible permeability can be made. Details are given of a method of low pressure ultrafiltration making use of these membranes. The method has been used to determine ultrafiltrable calcium in bovine blood-serum.

I wish to express my thanks to Mr G. L. Peskett for helpful suggestions.

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CCXL. THE HEAT-INACTIVATION OF CRYSTALLINE PEPSIN; THE CRITICAL INCREMENT OF THE PROCESS.

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(Received September 18th, 1933.)

IN a recent series of publications Northrop [1930, 1, 2; 1931] has advanced very strong evidence that pepsin is a protein. Starting from commercial pepsin, by suitably regulating the p_H and precipitating with the aid of magnesium or ammonium sulphate a crystalline product was obtained which had the general properties of a protein. In particular, solutions of this crystalline pepsin were coagulated by heat, and Northrop states that the inactivation of the enzyme either by heat or by alkali is quantitatively proportional to the denaturation of the protein. A successful effort was made [1931] to reactivate denatured inactivated pepsin along the lines used by Anson and Mirsky [1929; 1931, 1, 2] for the reversal of the denaturation of certain proteins, and the product obtained was identical with the original freshly prepared material, both in general properties and in its specific proteolytic activity. It seems safe to conclude therefore that the crystalline pepsin is in fact a protein. More recently Northrop [1932, 2] has shown that trypsin can similarly be obtained in a crystalline form and has the properties of a protein, whilst Sumner [1926] claims that the crystalline urease prepared by him is also a protein.

Work has been proceeding in this Department for a number of years with a view to determining the mechanism of the process of the denaturation of proteins. In this connection the conclusions mentioned above are of great importance, for if the processes of inactivation and denaturation of the crystalline enzymes do go hand in hand, then an investigation of the heat-inactivation of the enzymes might yield valuable information regarding the mechanism of the process of denaturation, especially as the rate of inactivation can be determined experimentally with great accuracy by a number of different methods. Consequently an investigation of the heat-inactivation of crystalline pepsin as prepared by Northrop was undertaken, and the results obtained are given in the present communication.

EXPERIMENTAL.

The crystalline pepsin used in the present investigation was prepared from Parke Davis pepsin u.s.p. 1:10,000 in the manner described by Northrop [1930, 1] using magnesium sulphate. The product was then purified by several recrystallisations in the manner given by Northrop, and the material thus obtained was tested exhaustively as described below, to ensure that it was identical with that obtained by Northrop himself.

When viewed under a microscope the pepsin was found to consist of transparent, colourless dihexagonal bipyramids, the slight yellow colour observed in the first crop of crystals having gradually been removed in the recrystallisation process. After drying, the crystals dissolved with difficulty, and the solution obtained was coagulated by heat. The pepsin was precipitated on

saturating the solution with either magnesium or ammonium sulphate. Positive results were obtained for the biuret reaction and for the xanthoproteic reaction. Polarimetric measurements gave a value for $[\alpha]_D^{25}$ of -70.5° compared with Northrop's values of between -68° and -72° . The nitrogen content as obtained by several micro-Kjeldahl determinations was 15 %. Reference to Northrop's paper [1930, 1] will show that all these tests are in agreement with those obtained using his preparations. It must also be shown, however, that the writer's pepsin has the same proteolytic activity as that of Northrop's preparation.

The method recommended by Northrop for its accuracy and rapidity and selected by the author is that in which a given quantity of the enzyme is allowed to act on a caseinogen solution, and the rate of hydrolysis is followed by measuring the change produced in the viscosity of the protein solution at various time intervals.

Briefly, the method consists of adding different known weights of pepsin dissolved in a given volume of water to a known volume of a caseinogen solution and measuring the viscosities of the mixtures at definite time intervals. From the results obtained a series of viscosity-time curves are plotted and from these the gradients at zero time are measured. From the gradient of the curve it is a simple matter to obtain the percentage decrease in viscosity produced per minute, and the arbitrary unit of activity is then described as this value divided by the number of mg. of pepsin-nitrogen present in the mixture. The value of this unit should of course be the same (within the limits of experimental error) independently of the curve from which it was obtained. The mean value of the unit obtained from determinations carried out with five different concentrations of pepsin was 1350. Similar treatment when applied to four curves given by Northrop [1932, 1] gave a value of 1250. As a considerable experimental error is possible both in calculating the time interval after mixing the pepsin and protein solutions and also in the extrapolation of the viscosity curves to zero for the measurement of the gradient, this agreement may be taken as being satisfactory. Furthermore the value 1350 agrees with the various values obtained by Northrop from different preparations over a number of years. It may therefore be taken that the activity of the writer's preparation as measured by the caseinogen viscosity method is the same as that of the various Northrop preparations. A further indication that the activities are identical will be given in a later section. For the present, however, it is sufficient to say that a study of the properties of the pepsin used in the present investigation has shown them to be identical with the properties of the crystalline pepsin prepared by Northrop.

Determination of the pepsin unit by the alcohol titration method.

From what has been said above it will be seen that although the caseinogen viscosity method for measuring the activity of the pepsin is comparatively rapid, it is at the same time not particularly accurate. Furthermore the interpretation of the figures obtained is obscure as no definite chemical significance can be attached to them. It is therefore advisable to use some other method which, although perhaps not so convenient, does at least have a chemical meaning. One such method is the formaldehyde titration method. Another similar method is the alcohol titration method of Willstätter and Waldschmidt-Leitz [1931].

This method is somewhat tedious and requires great accuracy. It serves, however, to give a measure of the number of carboxyl groups liberated by the pepsin in a given time and therefore is not subject to the same criticism as the viscosity method given above. This method was examined by the writer, and the following figures which were obtained for the pepsin unit will serve as an indication of its accuracy.

The optimum p_H for the hydrolysis of caseinogen by pepsin is given by Northrop as 1.8. Consequently it is at about this p_H that the alcohol titration method will be most sensitive. For the purpose of obtaining this p_H a citrate buffer was used. The final mixture was as follows: 5.5 cc. $M/3$ sodium citrate, 34.5 cc. $M/3$ HCl and 10 cc. water. The caseinogen solution used was prepared by the addition of 100 cc. 0.05 N ammonia to 6 g. Kahlbaum-Hammarsten

caseinogen, stirring and warming to 40° until all the caseinogen dissolved. 15 cc. of this solution were run slowly into 10 cc. of the buffer with constant shaking. By this means, although the caseinogen first precipitated slightly, it then redissolved to give a clear solution with just a slight blue colour. The requisite amount of pepsin was weighed out and dissolved in 10 cc. of the buffer. The two solutions were placed in the thermostat at 35°, and when temperature equilibrium had been established 5 cc. of the pepsin solution were measured into the caseinogen solution. A 10 cc. sample of the reaction mixture was immediately withdrawn and run into a known volume of alcoholic caustic soda solution sufficient to stop the reaction by attaining p_H about 8. After exactly one hour a second 10 cc. sample was also withdrawn and treated similarly. 1 cc. of thymolphthalein solution was added to each of the samples which were then titrated with the same alcoholic caustic soda solution as was used to stop the reaction until the faint blue colour of the indicator appeared ($p_H = 9.5$). 100 cc. of boiling alcohol were then added causing the blue colour to disappear and at the same time precipitating the caseinogen in a finely divided state. The titration was continued until the blue colour reappeared, showing that the titration of the protein was complete. The increase in alkali titration due to the hydrolysis of the caseinogen by the pepsin was noted. The p_H of the reaction mixture was also noted during the course of the reaction, and was always in the neighbourhood of 1.9.

A large number of determinations was made using different concentrations of pepsin and the figures obtained are collected in Table I, and are plotted as a

Table I. *Amount of hydrolysis produced in a caseinogen solution under the conditions specified above by different concentrations of crystalline pepsin.*

p_H of reaction mixture = 1.9.
Time of reaction, 1 hour at 35°.

Weight of pepsin present in reaction mixture (mg.)	Increase in titration (cc. N/50 alc. NaOH solution)	Weight of pepsin present in reaction mixture (mg.)	Increase in titration (cc. N/50 alc. NaOH solution)
1.2	1.03	4.8	3.67
1.9	1.62	5.7	3.79
1.9	1.78	5.7	4.00
3.0	2.65	7.6	4.43
3.0	2.80	7.6	4.66
3.3	3.00	8.9	4.69
3.8	3.24	10.8	5.12
4.1	3.52	18.6	6.05
4.5	3.60		

curve in Fig. 1. It is intended to use this curve as a standard in the subsequent work on the heat-inactivation in a simple manner to be described later. It should be noted that the values given were obtained with several different preparations, yet all lie on a smooth curve.

It will be seen from the figure that the hydrolysis produced is directly proportional to the concentration of pepsin at low concentrations, but at higher concentrations the rate of hydrolysis produced is less than it would be if the linear function continued to hold.

A series of determinations was carried out on Parke Davis pepsin to obtain comparative data between the activity of the newly-prepared crystalline pepsin and that of the commercial material. This comparison is of interest since it affords confirmation of the comparability of the activity of the writer's prepara-

tion with that of Northrop, even though Northrop did not use the alcohol titration method.

From the figures obtained it is possible as will be seen below to give a pepsin unit expressed in an analogous way to those already published by Northrop.

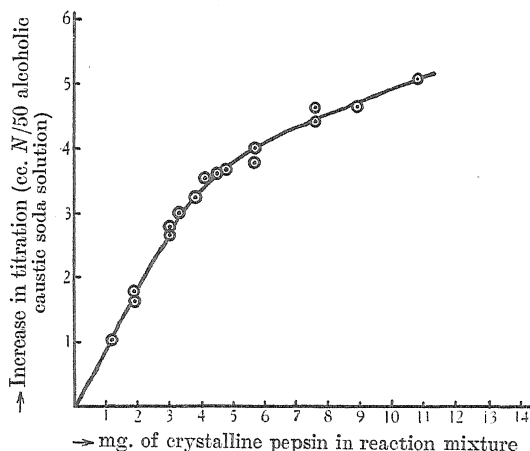


Fig. 1. Variation of activity with concentration of crystalline pepsin.

From the gradient of the curve at zero time one finds that 0.002 g. of pepsin in the reaction mixture liberates in one hour an amount of carboxyl groups requiring 1.8 cc. *N*/50 alcoholic caustic soda for neutralisation, *i.e.* $1.8 \times 1/50$ milli-equivalents are liberated per hour, per 2 mg. pepsin in the reaction mixture, or per 0.3 mg. pepsin-nitrogen. Hence the activity [*PU*] per mg. pepsin-nitrogen, may be represented

$$[PU] = \frac{1.8}{50 \times 0.3} \\ = 1.20 \times 10^{-1}.$$

A rough experiment with Parke Davis pepsin gave the following figures: 0.024 g. pepsin in the reaction mixture corresponded to a titration of 3.95 cc. *N*/50 NaOH.

From which

$$[PU] = 2.2 \times 10^{-2}.$$

Hence

$$\frac{\text{Activity of own preparation}}{\text{Activity of Parke Davis}} = \frac{120}{22} \\ = 5.5.$$

Northrop, using the caseinogen-formaldehyde method for obtaining the activity of his preparations gives the values 14 units for his own preparations, and 2.5 for Parke Davis pepsin, a relative activity of 5.6. This affords further evidence that the writer's pepsin is identical with that of Northrop.

The heat-inactivation of crystalline pepsin.

Although the process of heat-inactivation of the crystalline pepsin was not examined in any detail by Northrop, he performed one or two experiments with the object of determining whether the process followed the course of a simple unimolecular reaction. The conclusion drawn from the results obtained was that the reaction did follow the theoretical unimolecular course quite closely, thus

confirming the conclusion reached earlier by Tammann [1895] and Arrhenius [1907]. This result is confirmed in the present work, and unimolecular constants have been obtained for the reaction at various p_H values and at different temperatures.

The method of following the inactivation reaction used by the writer was as follows. About 0.03 g. of pepsin was dissolved in distilled water, and the solution was made up to 30 cc. with a solution of HCl of such a concentration as to give the required p_H . The solution was placed in a thermostat at 65°, and after temperature equilibrium had been established 7 cc. were withdrawn and run into a thin-walled test-tube immersed in an ice-water mixture, the time of this action being noted. After suitable time intervals further 7 cc. samples were withdrawn and the reaction stopped in the same way. The activity of each of the samples was then obtained by the alcohol titration method as before, but certain slight alterations had to be made in order to obtain conditions which were identical with those used in obtaining the standard activity curve.

In the first place the pepsin was now dissolved in water or a very dilute HCl solution whereas before it was dissolved in the buffer solution. To obtain therefore the same reaction mixture as before the following quantities and concentrations were used. The caseinogen solution used was 6.923 %, and the buffer solution contained 5.5 cc. $M/3$ sodium citrate to 34.5 cc. $M/3$ HCl, but no water was added. 13 cc. of the caseinogen solution were slowly run into 12 cc. of the new buffer solution with stirring, and when the pepsin solution (5 cc. unbuffered) was added it will be seen that the reaction mixture was identical with that used previously. The only difference was in the small concentration of HCl added with the pepsin which had been used to adjust the p_H for the inactivation period, but this was so small compared with the acid present in the buffer that it had no appreciable effect on the p_H of the reaction mixture used for the activity determination. The remaining 2 cc. of pepsin solution were used for the p_H determination by the quinhydrone method of Corran and Lewis [1924] at 25°.

As before 10 cc. samples were withdrawn at the beginning of the reaction and after exactly one hour at 35°. The increase in titration was measured, and from the activity curve the quantity of active pepsin present in the reaction mixture which corresponded to this increase was read off. The concentration in the initial sample was taken as 100 % and the percentage inactivation at each time interval was calculated. From each of these values it was possible to obtain a value of the unimolecular reaction velocity constant from the equation

$$k_{\text{uni}} = \frac{2.303}{t} \log_{10} \frac{a}{a-x},$$

where a = concentration of active pepsin at zero time (100 %); $a-x$ = concentration of active pepsin remaining at time t (% activity at time t); t = time in secs.

The fact that concordant values for k_{uni} were obtained showed that the heat-inactivation was indeed following the unimolecular reaction rate. Values of k_{uni} were obtained at 65° for a series of different p_H values, and the figures obtained are given in Table II.

From these figures it is seen that the effect of p_H on the rate of inactivation is considerable except between p_H 3.0 and 4.5 between which values the curve shows a fairly flat minimum. On the acid side of p_H 3, and the alkaline side of p_H 4.5, however, the rate increases exceedingly rapidly and in fact at p_H 6.5 the rate is too great to be measured. The approximate value given for p_H 6.9 was obtained as follows. The reaction mixture was prepared in the usual way and

Table II. *Heat-inactivation of crystalline pepsin at 65°.*

p_H	Mean value of $k_{uni} \times 10^4$	$\log_{10} k_{uni}$
2.30	19.9	3.2989
2.56	5.2	4.7160
2.65	4.7	4.6721
2.98	2.2	4.3424
3.30	2.1	4.3222
4.04	(4.0)	(4.6021)
4.50	3.6	4.5563
4.60	3.3	4.5185
5.43	15.2	3.1818
6.90	> 700.0	2.8451

It will be noted that 0.03 g. of pepsin in 30 cc. is equivalent to 5 mg. in 5 cc., *i.e.* the initial weight of pepsin in the reaction mixture is 5 mg. Reference to Fig. 1 shows that this is at the head of the steep straight portion of the curve so that as the pepsin becomes inactivated a rapid change in titration will be observed. In short, using this concentration causes the method to be particularly sensitive in measuring the degree of inactivation produced.

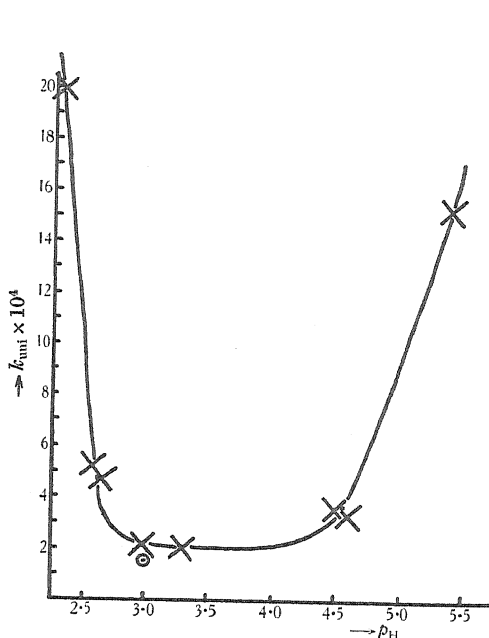


Fig. 2.

Fig. 2. Heat-inactivation of pepsin. Variation with p_H of k_{uni} at 65°.

○ Represents value obtained by Northrop.

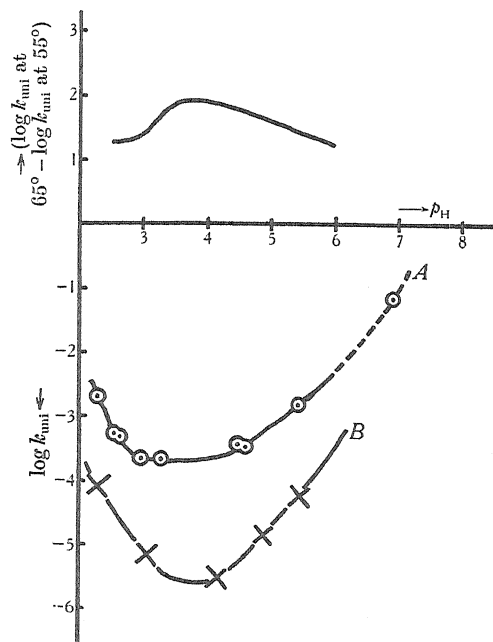


Fig. 3.

Fig. 3. Heat-inactivation of pepsin. Variation with p_H of $\log k_{uni}$.

○—○ Variation with p_H of $\log k_{uni}$ at 65°. ×—× Variation with p_H of $\log k_{uni}$ at 55°.
— Variation with p_H of $(\log k_{uni}$ at 65° - $\log k_{uni}$ at 55°).

placed in the thermostat at 65°. A sample was removed and cooled 3 minutes later. The activity determination gave an increase in titration of 0.05 cc. 0.073 N NaOH showing that the pepsin was almost completely inactivated by this short heating. Making a calculation on the assumption that maintenance for

1½ minutes at 65° has produced something over 95 % inactivation it is seen that the value of k_{uni} must be greater than 7×10^{-2} at this p_{H} .

The figures in Table II are plotted in Fig. 2 together with one value of k_{uni} obtained by Northrop, *viz.* 0.58 at 65° and p_{H} 3.0 (calculated with time measured in hours), *i.e.* 1.6×10^{-4} if t is measured in seconds. It is seen that this point lies very near the writer's curve. In Fig. 3 the curve is given in the form $p_{\text{H}} - \log k_{\text{uni}}$ to enable a wider range of p_{H} to be shown, and also for the purpose of showing how the critical increment of the process varies with p_{H} .

Effect of temperature on the heat-inactivation process.

To examine the effect of temperature, a second series of determinations of the values of k_{uni} for various p_{H} values was carried out exactly as described above but at a temperature of 55°. Since the technique is identical it is sufficient merely to give the data obtained; these are shown in Table III.

Table III. *Heat-inactivation of crystalline pepsin at 55°.*

p_{H}	$k_{\text{uni}} \times 10^5$	$\log_{10} k_{\text{uni}}$
2.30	8.2	5.9138
3.06	0.7	6.8451
4.15	0.3	6.4771
4.89	1.5	5.1761
5.42	5.4	5.7324

The behaviour with respect to alteration in p_{H} is therefore the same at 55° as it is at 65°. There is a flat minimum between p_{H} 3.5 and 4.5 and on either side of these values a sharp rise. The similarity is brought out by plotting the values in the third column ($\log_{10} k_{\text{uni}}$) against p_{H} in Fig. 3 on the same scale as the values obtained at 65°. The use of this diagram will be mentioned later.

The critical increment of the heat-inactivation process.

Consideration of the figures in Tables II and III shows that the process of heat-inactivation is one which possesses a very high temperature coefficient. This point is emphasised when the critical increment is calculated from the integrated form of the equation

$$\frac{d \log k}{dT} = \frac{E}{RT^2},$$

$$\text{viz.} \quad 2.303 (\log_{10} k_1 - \log_{10} k_2) = \frac{E}{R} \left(\frac{T_1 - T_2}{T_1 T_2} \right),$$

where k_1 and k_2 are unimolecular constants at temperatures T_1 and T_2 , E is the critical increment and R the gas constant.

The value in the bracket on the left-hand side of this equation is obtained at any given p_{H} by the difference in the ordinates of the two curves in Fig. 3. For the sake of simplicity a third curve has been drawn in Fig. 3 which represents at each p_{H} the difference between the ordinates of curves *A* and *B*. If we represent this difference by d , then the critical increment at any given p_{H} is given by

$$E \text{ (cals.)} = \frac{2 \times 2.303 \times d \times (328 \times 338)}{10},$$

i.e. $E \propto d$.

Consequently from the diagram we see that E is maximum at p_{H} 3.7 to 4.0 and falls off on both sides of this value until on the acid side of p_{H} 3 and the alkaline side of p_{H} 5.5 it attains an approximately constant value which is about the same in both cases. To bring this out more clearly several values of the critical increment have been calculated and are appended in Table IV.

Table IV.

p_H	Critical increment (55 to 65°)	p_H	Critical increment (55 to 65°)
2.3	71,000	4.5	91,000
2.7	68,000	4.9	80,000
3.0	73,000	5.43	74,000
3.5	88,000	6.0	69,000
4.15	96,000	6.5	67,000

These figures cannot be taken as absolute but they are probably correct to within ± 5000 , and the variation with p_H is probably a real one. It is quite certain, however, that the critical increment of the heat-inactivation process may be taken as being of the order 80,000 cal.

DISCUSSION OF RESULTS.

Let us consider in the first instance how the results obtained for pepsin agree with those already obtained by various workers for other enzymes. The process of the heat-inactivation of pepsin has been found to be one which follows the theoretical unimolecular course. Other enzymes for which the heat-inactivation process has been shown to be unimolecular are trypsin by Pace [1930], pancreatic lipase by McGillivray [1930], enterokinase by Pace [1931, 1], trypsin-kinase by Pace [1931, 2], pancreatic proteinase by Pace [1931, 3], and pancreatic amylase by Giri [1932].

The figures given in Tables II and III show that for pepsin the rate of heat-inactivation is minimum and almost constant over a p_H range from about 3 to 4.5. As the p_H is altered away from this range, however, the rate of inactivation increases rapidly for comparatively small alterations in p_H . Similar behaviour for the alteration in the rate of heat-inactivation with p_H is reported for yeast saccharase by Euler and Laurin [1919] the minimum rate being at p_H 4 to 5.

In the case of trypsin, a new method of preparation has been developed by Northrop [1932, 2] which gives a very pure crystalline trypsin having the properties of a protein. The rate of inactivation for the trypsin prepared in this way is minimum at p_H 1.8.

It is therefore seen that although the general behaviour of the rate of heat-inactivation with respect to change in p_H is similar in all these cases, it is not identical, the minimum rate obtained in the different cases being at different p_H values. In the case of the heat-denaturation of oxyhaemoglobin and egg-albumin (the proteins upon which the most reliable investigations have been made) it has been shown by P. S. Lewis [1926, 1, 2] that the rate of heat-denaturation is minimum at the neutrality point of water p_H 6.8.

It may be noted in passing that for neither process does the isoelectric point of the protein or the enzyme appear to have any special importance.

A striking similarity between the two processes, *viz.* protein denaturation and enzyme inactivation, is found in the magnitude of the critical increments. The value obtained for crystalline pepsin above is of the order 80,000 cal., agreeing with the value 75,000 given by Arrhenius [1907]. A collection of such data made by Haldane [1930] for other enzymes shows values ranging from 26,000 for lipase to 189,000 for peroxidase. The critical increment for the denaturation of oxyhaemoglobin was found by Lewis [1926, 1, 2] to be 77,000, and for egg-albumin 130,000 cal., so that the unusually high values are common to both processes.

A further point observed in the present investigation is that the critical increment varies with p_H , the value being maximum, as one would expect, at the range of p_H for which the rate of inactivation is minimum. In general, other investigators have not determined how the critical increments have varied with p_H , but have been content to obtain a mean value which gave them only the order of magnitude of the critical increment. For yeast saccharase, however, Euler and Laurin [1919] have shown that the critical increment is maximum at the p_H range over which the rate of inactivation is minimum and falls off rapidly as the p_H is altered away from this range. Booth [1930], working in this Laboratory, has shown, by examination of the denaturation results of Lewis [1926, 1, 2] and Cubin [1929] supplemented by further experimental determinations, that the critical increment of the heat-denaturation of oxyhaemoglobin behaves similarly with respect to change in p_H , being maximum at the p_H for which the rate of denaturation is minimum (6.8), and decreasing as the p_H is altered away from this value.

The comparisons made above therefore show that not only does pepsin behave as a protein, thus confirming the conclusion of Northrop, but that the heat-inactivation of pepsin is analogous to the heat-denaturation of egg-albumin or oxyhaemoglobin.

SUMMARY.

1. Crystalline pepsin has been prepared by the method of Northrop, and tests have been made to ensure that the product obtained was identical with that of Northrop both in general properties and in specific proteolytic activity.

2. An activity-concentration curve for this pepsin has been obtained using the alcohol-titration method of Willstätter and Waldschmidt-Leitz, and this curve has been used as a standard in the subsequent investigation of the heat-inactivation process.

3. The process of heat-inactivation is found to follow the theoretical unimolecular course.

4. At a given temperature the rate of heat-inactivation varies with p_H , being minimum at a p_H range from 3 to 4.5 and increasing rapidly when the p_H is altered from this range towards either the more acid or alkaline regions.

5. The effect of temperature on the rate of heat-inactivation has been examined and is found to be very marked. The critical increment of the process is found to be of the order 80,000 calories.

6. The critical increment is found to vary with p_H , being maximum (96,000 calories) at the p_H range for which the rate of inactivation is minimum and decreasing to a much lower value (70,000 calories) in the more acid and alkaline regions (p_H 2.3 and p_H 6.0).

7. The results obtained are compared with those obtained by other investigators for various enzymes and also with the results obtained for the heat-denaturation of proteins. It is shown that the two processes, the heat-inactivation of enzymes and the heat-denaturation of proteins, are analogous, in all but one point, *viz.* the location of the minimum speed, for which no explanation can be offered at present.

The above work was carried out under the direction of Prof. W. C. M. Lewis to whom the writer is indebted for help and advice.

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CCXLI. THE PHYSICO-CHEMICAL BEHAVIOUR OF LECITHIN.

III. THE ELECTROPHORETIC BEHAVIOUR OF LECITHIN-CHOLESTEROL DISPERSIONS.

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THE work described in this paper is a continuation of that commenced by H. I. Price and Lewis [1929] and extended by Jones and Lewis [1932].

The former measured, by means of a dynamic method, the surface tension of aqueous dispersions of pure lecithin as a function of p_H . It was found that a maximum value of the surface tension occurred at p_H 2.6 (approx.) in close agreement with the value of the isoelectric point (p_H 2.7) found by Fugii [1923-24] from distribution measurements¹. The surface tension falls to a very marked minimum on either side of the maximum referred to.

Jones and Lewis investigated the effect of the presence of pure cholesterol in the lecithin dispersion. It was found, using the same surface tension method as H. I. Price and Lewis [1929], that the addition of cholesterol, whilst leaving the general capillary behaviour of lecithin as a function of p_H unchanged, nevertheless introduces modifications of the surface tension values. The most important modification is a shift in the position of the maximum in the surface tension- p_H curve from p_H 2.6 to p_H 4.

This appears to indicate a corresponding shift in the position of the isoelectric point. But, as pointed out by H. I. Price and Lewis the evidence is not sufficient to make this certain. It is with this point, in the main, that the present work on the electrophoresis of lecithin-cholesterol mixtures deals.

EXPERIMENTAL.

Pure lecithin, prepared by the method of Levene and Rolf [1927], was used for these measurements.

Cholesterol itself does not disperse in water. It is, however, peptised by lecithin and is easily dispersed in the presence of this substance.

The method of preparation of dispersions of the mixed material was similar to that used by Jones and Lewis [1932]. Equimolecular weights of lecithin and cholesterol (approximately 2 g. of lecithin : 1 g. of cholesterol) were dissolved together in ether. The ether was then evaporated at room temperature. The whole of the mixture was dispersed by shaking with the correct quantity of water and the dispersion kept in an atmosphere of nitrogen. The dispersion was found to be quite stable and no separation of either of the constituents took place.

¹ The value of the effective isoelectric point of lecithin has since been determined directly by electrophoresis measurements and found to agree with the value p_H 2.7 [cf. C. W. Price and Lewis, 1933].

For the electrophoretic mobility measurements, aliquot parts of the above dispersion were diluted with citrate buffer to give a dispersion containing 0.05 % lecithin and the requisite proportion of cholesterol.

The apparatus and method of measuring the mobility and also the method of preparing and buffering the dispersions follow that recently described by C. W. Price and Lewis [1933]. The addition of 2 % of sucrose to the dispersions was found again to facilitate the adjustment and maintenance of a sharp boundary without affecting the mobility in any way.

The values of the velocities obtained in cm./sec. were corrected to 20° by multiplying by the factor η_x/η_{20} , where η_x and η_{20} are respectively the viscosities of water¹ at the temperature of measurement and at 20°.

As before, the p_H values below 7 were measured by the gold quinhydrone electrode [Corran and Lewis, 1924]; for higher values of p_H the glass electrode [Harrison, 1930] was used.

A representative record of an electrophoretic mobility determination is given:

Composition of the dispersion, 0.05 % lecithin; 0.025 % cholesterol (approx.); 2 % sucrose; $N/100$ citrate buffer.

Composition of the supernatant liquid: citrate buffer of the same ionic concentration and p_H (to within 0.01 p_H unit) as the dispersion.

Temp., 20.5°; $p_H = 5.87$; current, 3.0 milliamps; potential gradient, 2.80 volt/cm.

Movement of boundary:

Anode limb (upwards)	Cathode limb (downwards)	Time (mins.)
0.0	0.0	0
5.5	5.5	10
10.5	10.5	20
15.5	16.0	30

Mean movement, 15.75 scale divisions in 30 mins. (1 scale division = 0.043 cm.)

Electrophoretic velocity under a potential gradient of 1 volt/cm. at 20° = 13.0×10^{-5} cm./sec.

Table I shows the variation of the electrophoretic mobility with p_H of dispersions of mixtures of lecithin and cholesterol in equimolecular proportions.

Table I. *Showing the variation of electrophoretic mobility with p_H values of dispersions of mixed lecithin and cholesterol.*

(The sign denotes the sign of the charge.)

p_H	Electrophoretic velocity under a potential gradient of 1 volt/cm. at 20° cm./sec.
8.15	-28.5×10^{-5}
6.95	-23.6 "
6.48	-19.4 "
5.87	-13.0 "
5.22	-10.65 "
4.89	-9.9 "
4.05	-7.65 "
3.30	-4.6 "
2.51	+4.9 "
2.30	+10.4 "

¹ The ratio η_x/η_{20} for the solutions used is not sensibly different from that of water at the same temperature.

In order to compare the behaviour of the above mixture of lecithin and cholesterol with that of lecithin alone, the mobility- p_H curve for lecithin, reproduced from the above-mentioned publication of C. W. Price and Lewis, is plotted as a dotted line and the data of Table I as open circles in Fig. 1.

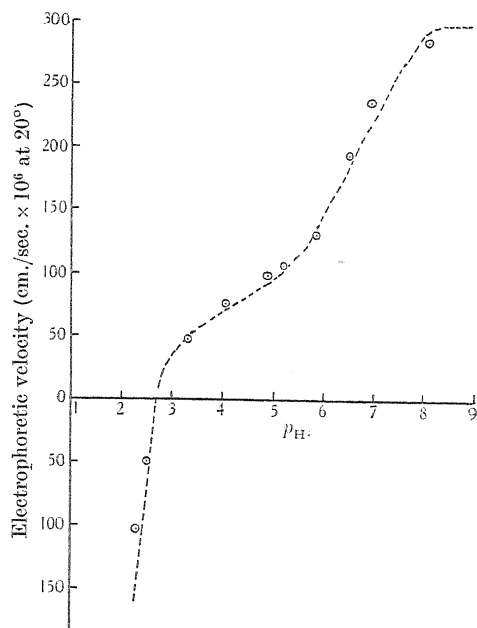


Fig. 1.

— — — Lecithin. ○ Lecithin and cholesterol.

From Fig. 1 it will be seen that the addition of an equimolecular proportion of cholesterol to lecithin has no effect on the electrophoretic behaviour of the latter substance.

DISCUSSION.

Combination of lecithin with glucosides, enzymes, cholesterol, proteins *etc.* has been described in the literature. It is not possible, however, in the majority of these cases, to differentiate between true molecular compounds, adsorption complexes (due to local interaction at certain positions on the mutual surfaces) or merely mechanical mixtures. Loewe [1912] considers that the majority of these so-called combinations are adsorption complexes.

Moravek [1927] claims to have shown that actual compounds are formed between lecithin and cholesterol and gelatin. No evidence, however, is available to show whether, in the absence of the third substance, combination between lecithin and cholesterol takes place, although the surface tension results of Jones and Lewis [1932] point to a union of some sort.

As previously pointed out, cholesterol will not form a dispersion in water. It is, however, peptised by lecithin and is easily dispersed in the presence of this material. It is quite possible, therefore, that cholesterol behaves merely as an "inert" body, its particles in a lecithin dispersion taking on the mobility of that dispersion in much the same way that an "inert" quartz particle in a protein

dispersion takes on the mobility associated with the protein itself. In other words, the two substances most probably exist together, in a dispersion, as an adsorption complex.

Further, it will be observed that no change in the position of the isoelectric point results from the addition of cholesterol to lecithin. It follows therefore that the points of union in the adsorption complex do not involve the amphoteric groupings of the lecithin or the groups which carry the charge due to adsorbed ions.

Clearly a shift in the position of the maximum in the surface tension- p_H curve does not necessarily imply a corresponding shift in the isoelectric point. This is borne out further by the effect of calcium chloride upon the surface tension maximum and electrophoretic speed respectively; the maximum being shifted towards the more acid region, the effective isoelectric point to the alkaline side.

SUMMARY.

The mobility- p_H relationship of dispersions of lecithin+cholesterol in equimolecular proportions has been determined. It is shown that no change in the mobility at various p_H values or in the position of the isoelectric point is produced by the addition of cholesterol to lecithin. It follows that the bonds or groups responsible for the formation of the lecithin-cholesterol complex cannot be the amphoteric groups of the lecithin or those on which ion adsorption takes place.

In conclusion the writer would like to express his thanks to Prof. W. C. M. Lewis, under whose direction this work was carried out.

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CCXLII. A SUBSTANCE INHIBITING BACTERIAL GROWTH, PRODUCED BY CERTAIN STRAINS OF LACTIC STREPTOCOCCI.

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AMONG the bacteria there are many instances where growth of one organism renders the medium unfavourable for growth of some other type of organism; but cases where an organism militates against growth of other strains of its own species, except by accumulation of the normal end-products of growth, are rare. The following account deals with an investigation into the biochemistry of two strains of lactic streptococci which produce during their growth some substance which very strongly inhibits the growth of other strains of lactic streptococci. The organisms were isolated from milk in the course of an investigation of trouble occurring during the manufacture of Cheddar cheese in a dairy factory [Whitehead and Riddet, 1933]. As is well known, development of lactic acid in the milk and curd is an essential part of the process of cheese manufacture, and the formation of acid can occur only if the lactic streptococci which are added as a "starter" culture grow readily in the milk and ferment the lactose. In the particular case under investigation most of the milk received at the dairy factory readily supported the growth of lactic streptococci and hence gave no trouble to the cheese-maker; but the milk from one particular source of supply proved entirely abnormal in that very little acid could be developed in it. The inhibitory action of the milk was so marked as to suggest the presence of some strong disinfectant, but none could be detected. Moreover, the bacteria present in the milk as contaminants were quite active, since added methylene blue was rapidly decolorised. The various types of bacteria present in the milk were isolated in pure culture, and it was found that there were present in large numbers some Gram positive cocci which, when allowed to grow in any sample of normal milk, rendered it unsuitable for the subsequent growth of lactic streptococci and hence prevented the development of acidity. Even when the cocci were destroyed by heat after they had grown for twelve or twenty-four hours the milk still retained its inhibitory power. Thus it became evident that the phenomenon was not due to an antibiosis between living organisms but to the production of some heat-stable substance which was inhibitory towards lactic streptococci.

Later, another Gram positive coccus which produced a similar effect was isolated from a sample of milk obtained from quite a different locality. There was a slight difference in this case in that the coccus itself produced appreciable quantities of acid. Thus milk in which this organism was growing appeared to allow acid production by lactic streptococci to proceed quite normally. When however the milk was pasteurised or sterilised, lactic streptococci were strongly inhibited just as was the case in the first example.

It was of interest therefore to classify these two unusual organisms and to attempt to determine the nature of the substance which they formed during their growth.

Nature and reactions of the two organisms.

Both organisms proved to be lactic streptococci. The only significant differences between them were in the rates of acid production in milk and the various sugars which they fermented. The first organism (9 S) produced acid very slowly, taking three days at 30° to clot milk; it had a tendency to grow in chains of six to ten units. The second organism (D 1) produced acid much more rapidly; milk was clotted in one day at 30° or in two days at 20°. Both organisms produced *d*-lactic acid from lactose and hence are included in the group of lactic streptococci according to the classification of Orla-Jensen [1919]. The organisms, like all lactic streptococci, were rather strict in their growth requirements; they grew only very slowly in the absence of a fermentable sugar from the medium. The best growth (as small clear colonies) was obtained on an agar containing whey from milk and an extract of yeast. Neither organism liquefied gelatin or reduced nitrate. Both organisms rapidly produced a reducing potential in milk at 37° as evidenced by the rapidity with which their milk cultures reduced methylene blue to the leuco-base. The sugar reactions were determined by growth in a peptic digest of caseinogen (0.5 % total N) containing 2 % of the various sugars; the time of incubation was 14 days at 20°.

Table I.

	Parts per thousand of lactic acid produced.									
	Glycerol	Xylose	Ara-binose	Rham-nose	Sor-bitol	Man-nitol	Fruc-tose	Glu-cose	Man-nose	Galac-tose
9 S	0.0	0.0	0.0	0.0	0.0	0.0	4.0	4.0	3.8	2.7
9 S	0.0	0.0	0.0	0.0	0.0	0.0	5.9	5.8	3.8	4.0*
D 1	0.0	0.4	0.4	0.4	0.0	0.0	5.8	5.2	5.2	3.4
	Sucrose	Maltose	Lactose	Raffinose	Inulin	Dextrin	Starch	Salicin		
9 S	0.0	0.4	4.5	0.0	0.0	0.0	0.0	0.0		
9 S	0.0	0.5	5.8	0.0	0.0	0.0	0.0	0.0		
D 1	6.7	3.6	5.9	0.0	0.0	2.9	1.3	3.8		

* Medium had 10 % yeast extract added.

According to Orla-Jensen's classification these reactions would indicate that 9S was a strain of *Strep. cremoris*. D1 resembled *Strep. mastiditis* in its reactions, except for its rather active habit of growth in milk. On the whole there was nothing to distinguish these two organisms from the majority of those present in the mixed cultures of lactic streptococci used as "starters" in cheese manufacture, except their ability to produce the inhibitory substance which had first drawn attention to them.

Method used for detection of the presence of the inhibitory substance.

The method used throughout the experiments for the demonstration of inhibition of acid production was as follows. 150 cc. of each milk to be tested were adjusted in temperature to 30°; 1 % of a twenty-four hour culture of lactic streptococci was added and the mixture was maintained at 30° for half an hour. 0.2 cc. of rennet solution was added, and after a further hour at 30° the clotted milk was cut into pieces about $\frac{1}{4}$ inch square. The mixture of curd and whey was incubated at 37° for two hours, and then the whey which had separated was drained off as completely as possible. The curd was kept at 30° for a further two hours, after which time the fresh lot of whey which had exuded was drained off and titrated with *N*/10 NaOH. A final titration was carried out

on the whey which drained from the curd during a further period of one hour at 30°. The two acidity values obtained were expressed as percentages of lactic acid. The figures obtained in this way give an indication of the total amount of acid formed by the streptococci during the period of the experiment; the difference between the figures gives a measure of the rate of acid formation.

The test described above is of course only of comparative value as between various samples of milk tested at the same time. Various factors (in particular, variations in the acid-producing power of the culture of streptococci used) make comparisons of tests carried out on different days impossible. Whitehead and Cox [1932] have indicated the technical applications of the above process as a means of making determinations of the activity or vitality of various cultures of lactic streptococci in several samples of the same milk. The process has been termed the "vitality test" and is of considerable use in the testing of "starters" used in the process of cheese manufacture. In the present instance the vitality test was used as a means of testing the inhibitory power of various milk samples, and of milk to which various additions had been made, on the production of acid by the same culture of lactic streptococci.

Influence of growth of Streptococcus 9 S on milk.

1. Fresh skimmed milk which had been steamed for half an hour was inoculated with a culture of 9 S in milk and allowed to stand at room temperature overnight. Next morning the milk still had an acidity within the range considered normal for fresh milk (actually 0.16 % as lactic acid) and contained approximately 50×10^6 cocci/cc. as determined by a direct count under the microscope. Methylene blue added to a sample of the milk was decolorised in half an hour at 37°. One portion of the milk was pasteurised at 63° for 30 minutes and a second portion was steamed for 30 minutes. Vitality tests were then carried out on the raw, pasteurised and steamed samples, and also on a portion of uninoculated milk, 1 % of a mixed culture of lactic streptococci being added to all the samples (Table II).

Table II.

Acidities as % lactic acid.

	Raw inoculated	Pasteurised inoculated	Steamed inoculated	Control
First acidity reading	0.16	0.13	0.15	0.33
Second acidity reading	0.18	0.11	0.15	0.54
Increase	0.02	-0.02	0.00	0.21

These results are typical of those obtained in many similar experiments. The apparent decrease in acidity in the pasteurised sample is not significant since the experimental error may on occasion amount to 0.05. The results indicate that the growth of 9 S had so influenced the milk as a medium for normal lactic streptococci that their power of producing acid was almost completely inhibited for a period of at least six hours. In some experiments the samples of curd were maintained at 30° for a further period of twelve hours. The acidity in the whey from the inoculated milk curds then reached a value of 0.8 to 0.9 % lactic acid as compared with a value of 1.1 to 1.2 % in the controls. Thus the action is inhibitory rather than bactericidal in nature. It is comparable with, though much more powerful than, the so-called bactericidal action of fresh milk and is evidently due to some heat-stable substance formed by 9 S during its growth, since the inoculated milk was still inhibitory even after it had been maintained at 100° for half an hour in order to destroy the bacteria.

2. In order to gain some idea of the concentration of inhibitory substance in a milk in which 9 S had grown, an experiment was performed in which various quantities of inoculated milk (containing on this occasion 26×10^6 of 9 S per cc.) were made up to 150 cc. with normal pasteurised milk (Table III).

Table III.

	Acidities as % lactic acid.								
	Percentage of steamed inoculated milk in normal past. milk				Percentage of past. inoculated milk in normal past. milk				Control normal past. milk
	10	20	30	40	10	20	30	40	
First acidity reading	0.32	0.29	0.23	0.20	0.26	0.20	0.16	0.15	0.33
Second acidity reading	0.60	0.47	0.33	0.26	0.47	0.30	0.21	0.18	0.54
Increase	0.28	0.18	0.10	0.06	0.21	0.10	0.05	0.03	0.21

These results indicate that relatively small quantities of the inhibitory substance are sufficient to produce a marked influence on the development of acidity. In some later experiments where more extensive growth of 9 S was permitted in the inoculated milk, quantities as small as 3 % of inoculated milk added to normal milk produced a mixture which was almost completely inhibitory to acid production. The results in Table III also indicate that the inhibitory substance is not completely heat-stable at 100° , for it is evident that there is a slight decrease in inhibitory power in inoculated milk which has been heated to that temperature.

Production of the inhibitory substance in media other than milk.

Several batches of medium were made consisting of a clear broth prepared by peptic digestion of sugar-free caseinogen to which were added 2 % of each of the five sugars which 9 S had proved capable of fermenting. After sterilisation, the media were inoculated with 9 S and incubated at 30° for four days. 25 cc. of each of the cultures were then made up to 150 cc. with normal pasteurised milk, and the mixtures were subjected to the vitality test. In every instance there was a complete inhibition of acid production, whereas in appropriate controls containing the uninoculated media, acid production was normal. 9 S gave practically no growth in the caseinogen broth alone and, as would be expected, produced no measurable amount of inhibitory substance. It was thus impossible to determine by an experiment such as this whether the substance was formed from any fermentable sugar or whether it was formed from protein material whenever growth of the organism was stimulated by the presence of a sugar.

Influence of the inhibitory substance on the increase in numbers of lactic streptococci in milk.

Although it seemed most likely that the results in the foregoing experiments were to be explained by inhibition of the growth of lactic streptococci, it was deemed desirable to determine whether this was actually the case or whether the streptococci were increasing in numbers without displaying their usual fermentative activity. Two identical samples of pasteurised milk were taken and to one was added 10 % of milk containing inhibiting substance; both were inoculated with 1 % of a twenty-four hour culture of normal lactic streptococci and incubated at 30° . At various intervals from the time of inoculation samples were taken, and the numbers of streptococci were estimated by direct count under the microscope according to the method of Breed and Brew [1916] (Table IV).

Table IV.

Time in hours	Numbers of streptococci in millions	
	Control	Experimental
$\frac{1}{2}$	22	17
$1\frac{1}{2}$	50	13
$3\frac{1}{2}$	285	20
$4\frac{1}{2}$	537	14
$6\frac{1}{2}$	804	14
10	—	200
12	1000	218
14	1000	436

The acidities of the control and experimental milks after fourteen hours were 0.84 and 0.52 % as lactic acid respectively. From these results it is evident that the inhibitory substance acts by delaying the multiplication of the lactic streptococci and not by interfering only with their fermenting power or otherwise modifying their habit of growth. The streptococci which finally grow in a medium containing the inhibiting substance have a normal appearance under the microscope.

*Partial isolation of the inhibitory substance and
determination of its properties.*

Two litres of sterilised skimmed milk were inoculated with 9S and allowed to stand overnight at room temperature. Next morning the milk contained approximately 170×10^6 cocci per cc. Vitality tests showed that 7 % of this milk culture added to normal milk was the minimum amount which would completely inhibit acid production by normal lactic streptococci for a period of six hours. Calcium chloride and rennet were added to the main bulk of the milk and the firm clot which formed was broken to allow the whey to separate. 1500 cc. of clear whey were obtained by filtration. Vitality tests showed that about 14 % of this whey added to normal milk gave a complete suppression of acid production by lactic streptococci. One litre of the whey was evaporated *in vacuo* at a temperature not exceeding 50° until its volume was reduced to 200 cc. This was poured into 800 cc. of absolute alcohol, and the bulky precipitate formed was filtered off. The filtrate was evaporated to dryness and extracted with absolute alcohol, and the insoluble material was again removed by filtration. The alcoholic solution was evaporated to dryness and the resulting glutinous residue (approximately 2 g.) was dissolved in 50 cc. of distilled water. Vitality tests showed that 2 % of this solution added to normal milk was the minimum amount necessary to inhibit acid production completely. Making allowance for the concentration, this corresponds to only 40 % of the original whey. Some of the inhibitory substance was shown to be present in the material soluble in 80 % alcohol but insoluble in absolute alcohol, so that it is possible that the substance (if it is a single entity) is not freely soluble in absolute alcohol. The concentrated solution of inhibitory substance, prepared as described above, was not inactivated when heated at 100° for 30 minutes.

In order to determine whether the substance were of protein nature some experiments were carried out to test the action of pepsin and trypsin upon it. The reaction of the concentrated extract was about p_H 4. A portion of it was adjusted to p_H 8 with NaOH to favour the action of trypsin. Portions of 5 cc. were treated according to the following scheme, then made up to 150 cc. with normal pasteurised milk and subjected to a vitality test (Table V).

Table V.

1.	5 cc. extract	
2.	"	+ 1/30 cc. 10 % pepsin solution
3.	"	"
4.	5 cc. adjusted extract	"
5.	"	"
6.	"	+ 1/30 cc. trypsin solution
7.	"	"

All the above tubes were incubated at 37° overnight, then tubes 3, 5 and 7 were pasteurised at 63° for 30 minutes.

Vitality tests on above samples made up to 150 cc. with normal pasteurised milk.
Acidities as % lactic acid.

	1	2	3	4	5	6	7	Control milk + pepsin	Control milk + trypsin	Control milk
First acidity reading	0.12	0.13	0.13	0.14	0.15	0.19	0.19	0.19	0.21	0.20
Second acidity reading	0.11	0.14	0.12	0.23	0.24	0.47	0.42	0.47	0.51	0.51
Increase	-0.01	0.01	-0.01	0.09	0.09	0.28	0.23	0.28	0.30	0.31

These results serve to define quite clearly the nature of the inhibitory substance. It is evidently a protein derivative (probably a polypeptide) which is readily destroyed by trypsin but is resistant to the action of pepsin. There is also an indication that incubation at 37° after adjustment to p_H 8 causes a weakening in power, probably due to a hydrolytic effect. In other experiments on similar lines, but in which the extract was heated to boiling-point after adjustment to p_H 8, the loss in power was greater, although with 9S the inhibitory action was never completely eliminated by this treatment.

Production of inhibitory substance by Streptococcus D1.

As has already been mentioned *Streptococcus* D1 differed from *Streptococcus* 9S in that it formed rather more acid during its growth in milk. Milk in which the organism had grown did not appear inhibitory to the growth of normal acid-producing streptococci unless D1 had first been destroyed by pasteurisation. Thus in an experiment on milk in which D1 had grown until its numbers amounted to 50×10^6 per cc., the results of vitality tests were as follows.

Table VI.

Acidities as % lactic acid.

	Raw inoculated	Past. inoculated	Control
First acidity reading	0.28	0.11	0.38
Second acidity reading	0.59	0.10	0.66
Increase	0.31	-0.01	0.28

Thus in the case of this organism either its own acid-producing power counter-balanced the loss in acid production due to inhibition of the normal streptococci, or else the inhibitory substance was not finally formed in the milk until the latter was heated. In most other respects the substance proved to have properties very similar to those of the substance produced by 9S. It differed mainly in its sensitivity to alkali. A concentrated extract of the substance in several experiments completely lost its power when it was adjusted to p_H 8 and incubated at 37° overnight.

Action of the inhibitory substances on pure cultures of bacteria.

The substances produced by both 9S and D1 were tested on various pure cultures. Both seemed to have a powerful action on the several cultures of lactic streptococci which were tested; neither restrained in any way the growth of a single strain of *Bact. coli* and of a strain of *B. subtilis*. The single instance up to the present where there has been a significant difference between them has been in their action on a strain of *Lactobacillus acidophilus*. The substance formed by D1 was markedly inhibitory towards growth of this organism whereas the substance formed by 9S was not. These observations need to be extended.

DISCUSSION.

It is evident from the foregoing results that the two strains of lactic streptococci described possess a property which has hitherto been unobserved with this species of bacterium. The significance of the formation of the inhibitory substances is evident in connection with any process in which lactic streptococci are used as biological agents for the development of acid. The formation of such substances in milk has already explained satisfactorily a difficulty which sometimes occurs during the manufacture of cheese. Moreover the reaction is of fundamental interest in the matter of the classification of the lactic streptococci. It remains to be seen whether the property of forming the inhibitory substance is a stable characteristic of these organisms. Up to the present time cultures in the laboratory, resown every two or three days in sterilised milk, have retained the property—in the case of 9S for seven months, and in the case of D1 for four months. The phenomenon would assume still greater significance if it were shown that normal lactic streptococci could ever acquire the property. Strains of streptococci vary so much in their properties from time to time that this is quite within the bounds of possibility.

If, however, these two strains of streptococci represent stable types the problem of their origin is of fundamental importance. Much attention has been devoted in the past to the question of abnormal milk which is inhibitory to the growth of lactic streptococci, since it influences the process of cheese manufacture so markedly. Leitch [1933] gave a comprehensive review of the matter and conducted experiments which indicated quite clearly that the inhibitory milk with which he was dealing was derived from cows suffering from udder disease, presumably streptococcal mastitis. Although he was of the opinion that the inhibitory factor was present in the milk as it came from the cow, it seems possible, in view of the fact that one of the streptococci described above is almost identical with *Strep. mastiditis*, that the inhibition observed by Leitch in milk might have been due to a formation of inhibitory substance by streptococci from the cow's udder, after the milk was drawn. Leitch also mentioned that he had obtained evidence that the inhibitory factor was associated with the milk-albumin. He did not give details of the evidence, but the suggestion that the inhibitory factor was of protein nature presents a further point of similarity with the present findings. The biochemistry of the streptococci which are the causative organisms in mastitis of the cow needs a more detailed examination before these points can be settled.

SUMMARY.

1. Two strains of lactic streptococci are described which produce during their growth substances having a marked inhibitory action on the growth of other lactic acid bacteria.

2. The nature of the inhibitory substances was investigated and evidence is submitted that they are of protein nature, possibly polypeptides, soluble in absolute alcohol and hydrolysable by trypsin and alkali but stable towards pepsin.

3. The significance of the properties of the organisms is discussed both from the point of view of the classification of the streptococci and from the technical standpoint, particularly with regard to processes like cheese manufacture in which lactic acid bacteria are used as a means for the production of acid.

This work was carried out with the assistance of a grant from the Empire Marketing Board.

The author wishes to thank Prof. W. Riddet and Dr F. H. McDowall for their suggestions and advice and Mr G. A. Cox for assistance with the experimental work.

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CCXLIII. THE METABOLISM OF CALCAREOUS ALGAE. I.

BY PAUL HAAS AND THOMAS GEORGE HILL.

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(Received October 10th, 1933.)

THE rather specialised conditions of growth to which the calcareous algae are subject as a result of a thick incrustation of calcium carbonate [Haas and Hill, 1933] provided the motive for the present investigation. The results here communicated are concerned only with the isolation of the galactoside floridoside and of a new pentapeptide of aspartic acid.

The material selected for examination was the red alga, *Corallina officinalis*, collected in the early summer of 1932 at Lyme Regis, and the results here described were all obtained from the one sample of about 9 kg. of wet material.

Preliminary experiments had shown that a concentrated hot water extract of the weed gave a biuret reaction and a positive Molisch reaction. At the outset a known weight of the dried material was dissolved in an excess of standard hydrochloric acid and by back-titration it was found that the amount of carbonate, calculated as CaCO_3 was of the order of 80 %. This figure was sufficient to indicate that considerable quantities of the weed would be required, and accordingly quantities of 1 kg. at a time of dried material were roughly broken up and extracted three times with hot water; the combined extracts were evaporated somewhat and then precipitated with basic lead acetate. The filtrate after removal of the lead with sulphuric acid was treated with sufficient baryta to remove all sulphuric acid and the solution, still acid with acetic acid, was evaporated under reduced pressure before precipitating with mercuric acetate. The bulky white precipitate (A) thus formed was filtered and washed and further treated as described below (p. 1803).

The filtrate from A was found to reduce Fehling's solution only after hydrolysis; the absence of pentoses and fructose was established by the usual colour tests, but on the other hand oxidation of the crude syrup obtained on evaporation yielded mucic acid, indicating the presence of galactose. The filtrate was accordingly evaporated under reduced pressure to a syrup (B). Prepared by this method the syrup contained considerable quantities of acetates, and it was not to be expected that a sugar would crystallise from this mixture. Extraction of the syrup with alcohol also yielded no crystallisable extract.

A different line of attack was therefore indicated, and this was provided by the work of Colin and Guégin [1930, 1, 2] who, extending the work of Kylin [1915], succeeded in isolating from *Rhodymenia palmata* a considerable quantity of a crystalline carbohydrate which, as in the case of the syrup B, did not reduce until after hydrolysis and also yielded mucic acid on oxidation. In a later paper these authors were able to show that this substance, which had been described by Kylin as trehalose, was in fact a galactoside of glycerol to which they gave the name of floridoside.

In view of the possibility that *Corallina* might likewise contain this substance it was decided to work up some fresh material following the procedure adopted by the above authors for isolating floridoside from *Rhodymenia*. Accordingly 250 g. of air-dried *Corallina* were extracted three times with hot water; the combined extracts were evaporated to about 200 cc. and treated with four times their volume of absolute alcohol; after some time the supernatant alcohol was poured off from a brown gummy deposit consisting of the peptide to be described later together with salts; the alcoholic solution was then evaporated to 200 cc. and the residue treated with eight times its volume of alcohol; after standing, the solution was filtered and once more evaporated under reduced pressure to a syrupy consistency; this was extracted with alcohol and the extract evaporated to dryness and kept in a vacuum desiccator. Repeated attempts to induce it to yield any crystals of floridoside were unsuccessful¹.

Having thus failed to isolate the carbohydrate by crystallisation from the syrupy extracts obtained by either of the two methods described, it was decided to try to isolate the substance by conversion into an acetyl derivative. With this end in view 2 g. of syrup were heated over a gauze with 4 g. of acetic anhydride and 2 g. of anhydrous sodium acetate, the mixture being continuously stirred until it just boiled, whereupon heating was at once stopped; the resulting viscous yellow-brown mass was allowed to cool and poured into water and extracted three times with ether. The ethereal extract, after washing free from acid with water and sodium carbonate, was dried and evaporated. A clear light yellow syrup, weighing about 0.6 g. resulted, which deposited crystals overnight; the latter were separated from the syrupy mother-liquors by stirring with a little cold alcohol and, after pressing on a tile, weighed about 0.2 g. This substance was soluble in hot alcohol, ethyl acetate, acetone, benzene or ether, but insoluble in light petroleum; it was recrystallised from a mixture of alcohol and light petroleum, from which it separated in aggregates of radiating prisms melting at 100–101°. (Found: C, 49.82; H, 6.04 %. Calc. for $C_{21}H_{30}O_{14}$: C, 49.80; H, 5.92 %.) These figures agree for the hexa-acetyl derivative of floridoside, a compound which had not been previously described; its specific rotation was $[\alpha]_D +108.5^\circ$, in acetone solution.

Isolation of floridoside.

In order to establish the fact that this substance was actually the acetyl derivative of floridoside, a larger quantity was prepared and de-acetylated by the method of Helferich and Brederick [1928]. For this purpose 1 g. of the hexa-acetate was dissolved in 7.5 cc. of dry chloroform and cooled to -15° ; to this were added, with constant shaking, 5 cc. of sodium methoxide whose titre had been previously determined with *N* sulphuric acid; the mixture, which set to a gel, was kept cold for $1\frac{1}{2}$ hours and then treated with the amount of *N* sulphuric acid which was equivalent to the sodium methoxide; after thoroughly shaking, the chloroform was run off from below and the aqueous alcoholic layer was evaporated to dryness in a vacuum desiccator. The residual mixture was extracted repeatedly with absolute alcohol, and the combined extracts on evaporation yielded a crystalline solid which separated from alcohol in wart-shaped aggregates and melted at 126–127°. (Found: C, 42.38; H, 7.12 %. Calc. for $C_9H_{18}O_8$: C, 42.52; H, 7.12 %.) A molecular weight determination by depression of freezing-point in aqueous solution gave 241. $C_9H_{18}O_8$ requires 254.

¹ On subsequently determining the amount of reducing sugar produced after hydrolysis of the syrup the floridoside content of the dry weed was estimated to be 0.09 %.

Colin and Guégin obtained for their sample a value of 245, by the method of plasmolysis.

The specific rotation was $[\alpha]_D +158.4^\circ$ as compared with 160° given by Colin and Guégin.

A portion of the pure substance when oxidised with nitric acid yielded crystals of mucic acid M.P. $213-214^\circ$. Another portion was hydrolysed by heating for 2 hours at 70° with 5 % hydrochloric acid; after neutralisation and evaporation the residual product was oxidised with bromine water, when on testing with α -naphthol and sulphuric acid it gave a characteristic green colour, indicating the presence of glycerol.

Taking all these facts into consideration it may be regarded as established that *Corallina officinalis* contains floridoside; the amount contained in this weed is, however, small and as it defied isolation by the method of Colin and Guégin, it is thought that the method of acetylation here described may be useful in other cases for establishing the presence of floridoside when only small quantities are present.

Examination of the precipitate A.

This material was suspended in water and decomposed with hydrogen sulphide: after removing the lead sulphide the filtrate was evaporated under reduced pressure at 40° ; a yellowish-brown syrup resulted which, on keeping for some weeks in a vacuum, dried to a friable resinous material D. The weight of the crude material obtained from 1 kg. of dried weed was about 8 g.

This substance was readily soluble in water but insoluble in organic solvents; its aqueous solution was neutral to litmus and gave a marked biuret reaction of a reddish-pink tint.

The material was hydrolysed by boiling in aqueous solution over a sand-bath with twice its volume of concentrated hydrochloric acid for $3\frac{1}{2}$ hours, after which the solution no longer gave a biuret reaction.

An estimation of amino-nitrogen gave a five-fold increase after hydrolysis, showing the substance to be a pentapeptide.

The nature of the product obtained on hydrolysis was determined as follows. 6 g. of the resinous substance D were dissolved in 10 cc. of water and boiled over a sand-bath with 10 cc. of concentrated hydrochloric acid for $3\frac{1}{2}$ hours. The dark brown liquid resulting was evaporated to dryness over a water-bath and left in a desiccator over caustic potash. The residue was then taken up in water, decolorised with charcoal and filtered; the filtrate, still strongly acid with hydrochloric acid, was treated with caustic soda until the reaction was brought to about p_H 3.6, when a precipitate appeared which was soluble both in acid and in alkali; it was filtered and crystallised from water from which it separated in prisms; heated in a sealed capillary tube the substance showed signs of melting with decomposition at about 270° .

Analysis showed the substance to be aspartic acid. (Found: C, 35.84; H, 5.26; N, 10.39 %. Calc. for $C_4H_7NO_4$: C, 36.08; H, 5.27; N, 10.53 %.) Aspartic acid was further characterised by the fact that a cold aqueous solution of this substance gave with a solution of copper acetate a deposit of fine blue needles of the sparingly soluble copper salt.

It is proposed to examine the properties of the peptide more closely when more material is available and also to investigate its physiological significance.

SUMMARY.

1. Extraction of the red sea weed, *Corallina officinalis*, with hot water yielded a solution containing floridoside and a new pentapeptide of aspartic acid.
2. The floridoside was isolated by conversion into its hexa-acetyl derivative M.P. 100–101°, a compound which has not been described before; this was then de-acetylated and the liberated floridoside was identified by analysis and by its physical constants.

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CCXLIV. STUDIES OF THE PHYSIOLOGICAL IMPORTANCE OF THE MINERAL ELEMENTS IN PLANTS¹.

VI. THE INFLUENCE OF POTASSIUM CHLORIDE ON THE RATE OF DIASTATIC HYDROLYSIS OF STARCH.

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(Received October 14th, 1933.)

THERE appears to be good evidence for the activation of animal diastases by a variety of salts, chlorides being the most effective [Haldane, 1930]. The results so far recorded for plant diastases are not so unanimous. Thus Sherman and Thomas [1915] reported an increased rate both of starch disappearance and of sugar production when numerous salts were added to malt diastase, but Sherman and Tanberg [1916] found no acceleration of sugar formation by the diastase of *Aspergillus Oryzae*, though Kellerman [1903] had already reported such an effect. These scanty and contradictory results suffer further from the fact that the acidity of the digests with which they were obtained was neither controlled nor observed, so that complete reinvestigation of the problem with all experimental precautions seemed the only means of reaching a satisfactory solution. This was undertaken as a contribution towards the study of the physiological importance of those elements which are derived by plants from the soil, and which remain in large part as simple salts within plant cells.

EXPERIMENTAL.

All the material used in this investigation was derived from a 1932 crop of potatoes var. King Edward. The potatoes were stored in a dark cellar and, for a short time before use, in the laboratory. All the experiments to be described were carried out during the months May-July 1933. At this late stage the tubers had produced small sprouts and were perceptibly softening due to loss of water. Large shoots 9-12 inches long with small leaves were obtained from some of the tubers by planting them in shallow boxes of sandy soil and keeping them moist. Three sorts of tissue were thus available and were used for the experiments, *viz.* tubers, small sprouts, and fairly large shoots.

Enzyme extracts were obtained from the tubers by peeling, mincing and finally pressing out the juice. During these operations the material was kept flooded with toluene to prevent bacterial contamination; this also had the further result of excluding air and so preventing blackening of the extract by oxidase actions which were otherwise very strong. The extract was allowed to

¹ Previous papers of this series have appeared in the *Ann. Bot.* 1930 *et seq.*, and the *New Phytologist* (in the press).

stand for half an hour, and the liquid could then be decanted free from suspended starch. A slightly different method was necessary with both the large and small shoots, which were ground vigorously with water (15 g. fresh weight with 25 cc. toluene and water) and the extract then filtered through muslin. Subsequent dilutions of the extract were made varying with the particular experiment to be performed.

Diastatic activity was examined in three different ways:

- (1) by the rate of disappearance of starch;
- (2) by the rate of formation of sugars from starch;
- (3) by the rate of formation of sugars from dextrin.

The rate of disappearance of starch was measured by Wohlgemuth's method carried out in narrow glass tubes enabling small quantities and relatively thin layers of solution to be used. This was necessary owing to the opacity of some of the enzyme solutions. The digests used contained 0.2 cc. of 0.5 % solution of Lintner's soluble starch + 0.05–1.00 cc. enzyme extract + 0.05 cc. toluene with water to make up to 1.25 cc. The tubes were incubated at 35° for a suitable time as determined by preliminary experiments; iodine was then added to each tube. The tube containing the weakest enzyme solution causing complete disappearance of the starch was then noted and the strength of the original enzyme extract calculated.

The rate of formation of sugars from starch was measured by the Hagedorn-Jensen method as modified by Hanes [1929]. Incubation was carried out again at 35°, the time period required usually being 24–48 hours. Preliminary experiments showed that these intervals lay on the linear portion of the hydrolysis progress curve. Lintner's soluble starch (supplied by B.D.H.) was again used as substrate. Three digests were used for every estimation.

Commercial preparations of dextrin could not be used for the third set of experiments as they were found to contain a strong inhibitor of the enzyme. No linear phase existed in the progress curves of their hydrolysis, which was very incomplete. The inhibitor could not be removed by electrodialysis or by controlling the p_H . Dextrin solutions were, therefore, prepared in the laboratory from Lintner's starch by the following method. A thick paste was prepared by dissolving 12.5 g. of starch in 250 cc. hot water, boiling for 10 minutes and cooling to 35–40°. The paste was strained through muslin while still warm, and 0.25 g. of an active takadiastase preparation (presented by Parke, Davies and Co.) added in 25 cc. of water. The progress of hydrolysis was followed by spot tests with iodine outside the solution. When the iodine colour had entirely disappeared, but the red dextrin colour was still present, the enzyme was destroyed by boiling for 2 minutes. The dextrin solution thus prepared contains a large amount of reducing sugars which carries the determination off the scale of the Hagedorn-Jensen method. This was removed by fermentation with an equal volume of 15 % yeast suspension for 3 hours at 35°. A stream of air was bubbled through the flask throughout the 3 hours. The yeast could not be removed by the usual method of flocculating with alumina cream since after such treatment the dextrin preparation inhibited the hydrolysing enzyme. Separation was effected by 2 minutes' boiling followed by centrifuging at about 3000 r.p.m. A clear solution of the dextrin with only slight reducing powers and no enzyme inhibitor could then be decanted. Using this solution as substrate the rate of sugar formation by the potato extract was determined as with the starch solutions except that the incubation time required was only 1 or 2 hours.

The action of potassium chloride on the enzyme activity was first examined by adding 5 cc. of 0–20 % potassium chloride to 5 cc. of enzyme extract and after a thorough mixing adding 2 cc. of this mixture to 50 cc. of substrate solution. In all the experiments recorded here the original concentration of potassium chloride used was 5 % since this amount was found to be optimum. The final concentration of potassium chloride was 0.19 % in the digests where sugar formation was examined. In the Wohlgemuth experiments in which the concentration of enzyme was made to vary the amount of potassium chloride was kept constant relative to the amount of enzyme. Control experiments were performed by adding 5 cc. of distilled water to the enzyme extract.

All the extracts were strongly buffered by the natural buffer systems at p_H 6, and the frequent colorimetric determinations carried out showed that this did not vary with the addition of

potassium chloride or any of the other experimental manipulations. The optimum p_H of the potato diastase was found to lie between 6 and 6.5. It was therefore unnecessary to add artificial buffers which inevitably complicate the system when salt effects are under investigation. Every precaution was taken by the use of toluene and by boiling the solution whenever possible to avoid bacterial infection.

In the following paragraphs the results are expressed in terms of the ratio:

$$\frac{\text{rate in presence of potassium chloride}}{\text{rate in absence of potassium chloride}}$$

This method is adopted as it conveys all the essential facts and avoids difficulties that would otherwise arise in expressing absolute rates in units applicable to all the experiments.

Tubers.

The rate of starch disappearance in the presence and absence of 5 % potassium chloride as measured by the Wohlgemuth method in the form already described showed an increase due to the salt. The ratio of the two rates of breakdown was about 1.8.

The corresponding rates of sugar formation, determined with the same enzyme extract, also showed an increase in the presence of the chloride. The ratios of the two rates were 1.97 (24 hours' digestion) and 1.85 (48 hours' digestion).

The Wohlgemuth results can only be regarded as very approximate but in view of later evidence the similarity of the ratios acquires a certain significance. Numerous repetitions of the sugar determination with further tuber extracts gave similar results.

The rate of sugar formation from dextrin was also examined and the results were in sharp contrast with the above. Two separate experiments each containing three digests gave the following results:

	Exp. 1	Exp. 2
$\frac{\text{Rate with KCl}}{\text{Rate without KCl}}$	1.03	0.98

There is therefore no activation of the enzyme causing the formation of sugars from dextrans.

Small sprouts.

Three experiments on the rates of starch breakdown by extracts of these shoots gave the following ratios:

	Exp. 1	Exp. 2	Exp. 3
$\frac{\text{Rate with KCl}}{\text{Rate without KCl}}$	1.5	1.5	1.7

Considerable activation took place, therefore, though probably not so much as in the tuber extracts.

The formation of sugars from starch on the other hand showed no acceleration due to potassium chloride. Experiments were performed with extracts from green shoots produced in the light and from aetiolated shoots formed on tubers kept continuously in the dark. The result was the same in both.

	Enzyme extract from green shoots	Enzyme extract from aetiolated shoots
$\frac{\text{Rate with KCl}}{\text{Rate without KCl}}$	1.00	1.02

This it will be noticed is in sharp contrast with the corresponding result for tuber extracts where 80–100 % activation took place.

The production of sugars from dextrin was not activated:

	Exp. 1	Exp. 2
$\frac{\text{Rate with KCl}}{\text{Rate without KCl}}$	0.95	1.00

Large shoots.

The values obtained for the ratio of the rates with and without potassium chloride were:

	Starch disappearance	Formation of sugar from starch	Formation of sugar from dextrins
	1.3	—	—
	1.1	1.3*	1.00
	1.5	—	0.85
	1.2	—	—
Mean	1.3	1.3	0.93

* Average of four titrations (two digests).

Activation is evident in the rate of starch disappearance and in the rate of formation of sugar from starch, but not in the formation of sugar from dextrin.

The amount of activation is less than in tubers or in younger sprouts, but it is noteworthy that, as with tuber extracts, the amount of activation of sugar formation is equal to the amount of activation of starch breakdown.

DISCUSSION.

It is clear from the above results that the diastatic hydrolysis of starch must be considered in at least two stages, each stage involving its own enzyme. One enzyme responsible for the immediate breakdown of starch molecules to form "dextrins" is capable of activation by potassium chloride (and perhaps other salts) while another enzyme concerned with the production of sugar from the dextrin formed by the first enzyme is not capable of such activation. This is true of all the material we have examined as may at once be seen from the foregoing section. The rate of formation of sugars from a starch substrate expresses the summation of these two processes and the results obtained here are at first sight less harmonious. Extracts from tubers and the larger shoots showed an activation but extracts from small shoots showed none. A simple explanation is provided, however, if we suppose that the tubers and large shoots contain relatively little of the starch-destroying enzyme and relatively much of the sugar-forming enzyme, and that the contrary relationship holds in the young sprouts. Then in the young sprouts where the starch-destroying enzyme is supposed to be comparatively plentiful an activation of this first enzyme will have no effect on the rate of sugar formation because it will still be restricted by the less active and inactivable second enzyme.

To clinch this argument it is necessary to show that the actual quantities of enzyme as shown by their activity in the presence of excess substrate have the appropriate relative values. To do this directly it would be necessary to convert the amounts of starch destroyed into their hexose equivalents, using some arbitrary factor for conversion, and to rely on the values supplied by the Wohlgemuth method. A more reliable comparison, having regard to the experimental data available, can be made by comparing the two methods of sugar production. When the first enzyme is more abundant and the "dextrinase" enzyme is controlling the rate of reaction the ratio

$$\frac{\text{Rate of sugar production from starch}}{\text{Rate of sugar production from dextrin}}$$

must be 1; conversely when the first enzyme is the less plentiful the ratio will fall below unity. The values calculated from the experimental results were:

Tubers	0.050
Small sprouts	1.149
Large shoots	0.118

and therefore provide striking verification for the theory.

All our results are thus explained by the fact that the starch-dissolving enzyme of diastase can be activated by potassium chloride but that the "dextrinase" component cannot. It is interesting to note that the apparently discordant results of earlier workers can all be explained in the same way. All the experiments performed with a method measuring the rate of starch disappearance indicated an activation by the salts employed [malt diastase, Sherman and Thomas, 1915; takadiastase, Sherman and Tanberg, 1916; potato diastase Hahn and Schweigart, 1923]. Apparently contradictory results were obtained, however, when the rate of sugar formation was taken as a criterion, and apart from possible deficiencies of technique, such as lack of control of acidity, these differences may well have been due to different proportions of starch-dissolving and sugar-forming enzymes in the preparations used by the different workers.

SUMMARY.

1. The effect of 5 % potassium chloride on the activity of potato diastase was examined in extracts of tubers, young sprouts and older shoots.
2. It was found that the rate of breakdown of starch was always accelerated, but that the formation of sugars from dextrin obtained from the starch was never accelerated.
3. In the presence of an excess of the substrate, the rate of production of sugars from starch was accelerated only if the rate of starch breakdown was slow relatively to the rate of formation of sugars from dextrin. This is to be expected from the previous results assuming that the dextrin is an intermediate product in the hydrolysis of starch to sugars. It furthermore affords a simple explanation of the apparent discrepancies between the results of previous workers.

This work was carried out as part of an investigation made possible by a grant in aid from the Department of Scientific and Industrial Research to whom our gratitude is due.

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CCXLV. THE MICRO-DETERMINATION OF PHOSPHORUS AS PHOSPHOMOLYBDATE.

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(Received October 17th, 1933.)

THE oxidation of organic matter with sulphuric and nitric acids and the estimation of ammonium phosphomolybdate volumetrically was introduced into biochemical work by Neumann [1902]. Plimmer and Bayliss [1906] found that the precipitation was not certain and the filtration was slow and liable to error. They defined the conditions for precipitation, introduced a new way of rapid filtration and proved the accuracy of the method for quantities of phosphorus ranging from 10 to 20 mg. Gregersen [1907] also determined the conditions of precipitation and found the method accurate for 1 to 30 mg. of phosphorus. Woerner [1908] confirmed the accuracy of the method. Other workers, Raper [1914], Heubner [1914], Jodidi and Kellog [1915], Kleinmann [1919], Iversen [1920], Euler and Svanberg [1921], Kuhn [1923], Sørensen [1925], Macheboeuf [1926] and Flatter [1933] have maintained either that the method is inaccurate for micro-quantities, or of no value for quantities below 0.1 mg. Greenwald [1913] and Taylor and Miller [1914] however obtained satisfactory results with quantities of 0.015 to 0.12 mg. Pregl [1930], who weighed the precipitate, regarded the process as accurate and quotes Lieb as proving the method applicable volumetrically to 0.03 mg.

Since excellent results are generally agreed to be given with from 0.1 to 10 mg., there seemed no reason for the failure with 0.01 to 0.1 mg. Having used the method for estimating 1 to 30 mg. since 1906, we were interested to ascertain the reason for the general failure with the micro-quantities.

EXPERIMENTAL.

It was shown by Plimmer and Bayliss [1906, p. 441] that the condition for complete precipitation of ammonium phosphomolybdate was the presence of 30 cc. of 50 % ammonium nitrate (17 g.) solution for 10 cc. of conc. sulphuric acid in a volume of about 200 cc. The same conditions were applied to the precipitation of micro-quantities of phosphorus from 0.01 to 1 mg., but with smaller quantities of reagents and in a smaller flask. In all experiments there was complete precipitation.

Filtration and washing of the macro-precipitate was accomplished on a filter-paper on a perforated platinum plate fused into a glass funnel. This filter has been found unsuitable for micro-quantities, even with two papers of barium sulphate quality. The use of asbestos on this filter was rejected as a large amount of inert material would be present during the subsequent titration with the necessity of testing the filter before each estimation. Trial of an asbestos filter of the form described by Bertrand [1913] for glucose estimation showed that it

retained the fine yellow precipitate, and that the latter could be dissolved on the filter in standard alkali and titrated in the usual way. Titration of the micro-quantities was carried out with $N/10$ or $N/20$ alkali and acid. The results with the micro-quantities of 0.1 mg. and less were generally from 5 to 10 % too high, just as had been found by other workers.

Numerous trials were made altering the amount of acid in the solution during precipitation of the ammonium phosphomolybdate. It was found that complete precipitation took place in concentrations of nitric acid from N to $5N$, and in concentrations of sulphuric acid from N to $3N$ in presence of the corresponding amount of ammonium nitrate. The results were still too high. Above $3N$ sulphuric acid, precipitation did not occur even on the addition of more ammonium nitrate.

Trials with alteration of the amount of ammonium molybdate in the solution gave irregular results, generally higher with more ammonium molybdate. Impurity in the ammonium molybdate was suspected as the cause of the high results. At this time Prof. J. L. Rosedale was testing Pregl's gravimetric method in this laboratory with which he obtained satisfactory results. Pregl used a special acid ammonium molybdate for precipitation. In its preparation it was noticed that a small yellow deposit was present in a larger bulk of white precipitate, which indicated the presence of small amounts of phosphorus in the reagent. On testing this reagent in place of the usual ammonium molybdate, the results were satisfactory. The cause of the high results was an impurity of phosphate in the molybdate reagent.

One other change was made in the procedure of Plimmer and Bayliss. The precipitate of ammonium phosphomolybdate was washed with 50 % alcohol, which was shown by Taylor and Miller not to dissolve it. The compound is slightly soluble in water.

The procedure of micro-estimation is as follows. To the measured volume of unknown solution in a 100 cc. flask are added 10 or 20 cc. of 10 % ammonium nitrate solution and 0.5 or 1 cc. of conc. H_2SO_4 . The mixture is raised to the boiling-point and from 1 to 10 cc. of Pregl's ammonium molybdate solution are added¹. Precipitation begins immediately and filtration can be carried out after 15 minutes. The acid solution is filtered through a Bertrand filter into a filter-flask, and the flask and filter are washed 6 times with 3-5 cc. of 50 % alcohol. It is not necessary that the whole of the precipitate be washed on to the filter. The filter-tube is removed, washed on the outside with water, and placed in one opening of a two-holed cork fitting the 100 cc. flask; the other opening carries a small bent tube for connection to a filter-pump. A slight excess of $N/10$ or $N/20$ alkali is run on to the filter from a burette; on stirring the precipitate dissolves and the solution is drawn into the flask, and the filter is washed 6 times with 3 to 5 cc. of water. The total volume is about 25 cc. A piece of porous plate is put in and the mixture is boiled for about 5 minutes to remove ammonia; one drop of phenolphthalein solution is added and the solution titrated with standard acid. A slight excess of the acid is added, the solution is again boiled to remove CO_2 and back-titrated with $N/10$ or $N/20$ alkali. The same factor, 1 cc. $N/20$ $NaOH = 0.1268$ mg. P_2O_5 or $= 0.05536$ mg. P ,

¹ Pregl's solution is made as follows. 150 g. of powdered ammonium molybdate are treated with 400 cc. of boiling water and shaken until dissolved. When cool, the solution is added slowly and with constant shaking to 50 g. ammonium sulphate in 500 cc. of nitric acid of sp. gr. 1.36 in a litre flask. After standing for 2 days the mixture is filtered and stored in a brown glass bottle. It has been found that 61 g. of ammonium nitrate can be used instead of the ammonium sulphate. The reagent slowly gives a deposit, and must be filtered before an estimation of phosphorus.

is used. Quantities from 0.01 to 0.1 and 1.0 mg. P have been accurately estimated as shown by the following figures:

mg. P taken	Titration cc. N/20	mg. P found	mg. P taken	Titration cc. N/20	mg. P found	mg. P taken	Titration cc. N/20	mg. P found
0.1	1.9	0.1052	0.09	1.75	0.0969	0.08	1.5	0.0830
	1.9	0.1052		1.55	0.0858		1.45	0.0803
	1.9	0.1052		1.6	0.0886		1.4	0.0775
	1.75	0.0969		1.5	0.0830		1.45	0.0803
	1.75	0.0969		1.6	0.0886		1.45	0.0803
0.07	1.3	0.0720	0.06	1.1	0.0609	0.05	1.0	0.0554
	1.3	0.0720		1.1	0.0609		1.0	0.0554
	1.2	0.0664		1.2	0.0664		1.0	0.0554
	1.2	0.0664		1.05	0.0581		1.0	0.0554
	1.25	0.0692		1.15	0.0637		1.0	0.0554
0.04	0.85	0.0471	0.03	0.5	0.0277	0.02	0.45	0.0249
	0.8	0.0443		0.6	0.0332		0.35	0.0194
	0.7	0.0388		0.6	0.0332		0.45	0.0249
	0.8	0.0443		0.6	0.0332		0.45	0.0249
	0.8	0.0443		0.55	0.0305		0.45	0.0249
0.01	0.2	0.0111	0.1	1.75	0.097		cc. N/10	
	0.25	0.0138	0.2	3.5	0.194			
	0.25	0.0138	0.3	5.1	0.282	0.7	6.0	0.664
	0.25	0.0138	0.4	7.05	0.390	0.8	7.0	0.775
	0.3	0.0156	0.5	8.75	0.484	0.9	7.7	0.852
			0.6	10.75	0.595	1.0	8.6	0.952

All the titrations were made with an ordinary burette. Better results would have been obtained with a micro-burette, and N/25 or N/50 alkali and acid could have been used.

Filtration, washing and titration can be effected in 15 to 20 minutes, so that four estimations can be made in 1 hour. For quantities above 1 mg. P, the perforated platinum plate is preferable.

The sensitivity of the precipitation of ammonium phosphomolybdate under the above conditions has been tested qualitatively. Quantities of P from 0.009 to 0.006 mg. began to come down in 2 to 3 minutes: from 0.005 to 0.003 mg. in 6 to 12 minutes; 0.002 mg. showed on standing 1 hour and 0.001 mg., which was just visible but quite evident on filtering on to the asbestos, in 1½ hours.

The method is applicable to solutions of inorganic phosphate as above described. It is applicable to all forms of organic phosphorus for estimation of total phosphorus. In this case, 1 cc. of conc. H_2SO_4 is added, oxidation is effected with nitric acid and to the cool residue 20 cc. of 10 % ammonium nitrate are added and then 5 to 20 cc. of Pregl's ammonium molybdate. Inorganic phosphate in the presence of phosphoric esters not rapidly hydrolysed by acid, such as are present in blood-filtrates, can be estimated, especially if filtration is carried out after 15 minutes, or if the precipitation is allowed to proceed at room temperature overnight, which was shown by Plimmer and Page [1913] to be quantitative. The conc. H_2SO_4 can be omitted if 10 cc. ammonium molybdate be added. The following determinations were made with 5 cc. of a trichloroacetic acid blood-filtrate:

- (1) 7.74 mg. per 100 cc. (hot) as against 7.44 mg. by Fiske and Subbarow's colorimetric method
 (2) 3.88 " (hot) " 3.34 " kindly performed by Dr Griffiths
 3.34 " (cold)

It has not been possible to test the method for a compound like phosphagen which is very easily hydrolysed by acid. For this purpose it would be necessary to precipitate the inorganic phosphate as ammonium magnesium phosphate, to add acid to the filtrate to hydrolyse the compound and then estimate the

inorganic phosphate. It would also be possible to make this estimation by dividing the solution into two parts, to add acid to one part and then estimate the inorganic phosphate in both portions as ammonium magnesium phosphate. The difference would give the phosphagen-phosphorus. The phosphorus in the ammonium magnesium phosphate can be estimated by the molybdate method after dissolution in dilute nitric acid.

SUMMARY.

The accurate estimation of micro-quantities of 0.01 to 0.1 mg. of phosphorus by the molybdate method depends upon (1) the conditions of precipitation; 20 cc. of 10 % ammonium nitrate solution must be present for every cc. of conc. H_2SO_4 ; (2) the use of a purified solution of ammonium molybdate such as that of Pregl, (3) the filtration of the precipitate on an asbestos filter such as that of Bertrand.

A research grant from Imperial Chemical Industries has covered the cost of materials used in these experiments.

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CCXLVI. STUDIES IN THE BIOCHEMISTRY
OF MICRO-ORGANISMS.

XXXV. THE METABOLIC PRODUCTS OF *BYSSO-
CHLAMYS FULVA* OLLIVER AND SMITH.

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(Received October 27th, 1933.)

OLLIVER AND SMITH [1933] described a new Ascomycete, *Byssochlamys fulva*, which is of fairly frequent occurrence as a cause of spoilage in processed fruits and is of considerable economic importance in the fruit canning industry. The source of the fungus is at present unknown, and the prevention of infection is difficult on account of the high resistance to heat shown by the spores. The mature conidia are unaffected by the normal sterilising process in which the maximum temperature attained exceeds 90°. In connection with its resistance to what would usually be considered lethal conditions it is of interest to note that the type species of the genus, *B. nivea* Westling [1909], can withstand prolonged immersion in 90 % alcohol and was actually isolated from botanical specimens preserved in spirit, the mould appearing as soon as a portion of the specimen became uncovered owing to gradual evaporation of the preservative.

The present paper is an account of the chief metabolic products which are formed when *B. fulva* is grown on a synthetic medium containing glucose as the sole source of carbon. In a preliminary test, in which the mould was grown on Czapek-Dox solution in test-tubes, it was noted that, after about 4 weeks' incubation, addition of mineral acid to the metabolism solution gave a precipitate which was soluble in ether. Further experiments show that the amount of precipitate obtained increases with continued incubation and appears to reach a maximum when the glucose is entirely consumed; also that the yield varies with the temperature of incubation, being less at 30° than at 24° and becoming nil at 37°. Large-scale experiments, in which the mould was grown on batches of 35 litres of medium at 24°, have resulted in the isolation, from the acid precipitate, of a new and specific mould product, $C_{18}H_{20}O_8$, M.P. 163.5°, which is definitely toxic to mice and for which the name *byssochlamic acid* is proposed. The substance titrates as a tetrabasic acid, giving salts of the type $C_{18}H_{20}O_8R_4$, and it is probable that it is contained in the metabolism solution as a mixture of salts of this general formula, acidification giving the free acid, $C_{18}H_{24}O_8$, which immediately loses two molecules of water to give $C_{18}H_{20}O_6$. It is interesting to note that Wijkman [1931] describes a mould product, glauconic acid II, which has the formula $C_{18}H_{20}O_6$ and titrates, on warming, as a tetrabasic acid. The melting-point of glauconic acid II, however, 186°, is sufficiently different from that of our product, 163.5°, to warrant our presumption that the two are different substances.

The main metabolic product of the mould is mannitol, formed in amounts corresponding roughly with 30 % of the total sugar utilised. Other substances are produced in small amounts and are precipitated along with the byssochlamic acid, but these have not yet been investigated. This is only one of many instances of the production of mannitol from glucose by mould fungi. It is now known that a fairly large number of species, belonging to several different genera (*Penicillium*, *Aspergillus*, *Helminthosporium*, *Clasterosporium*, and now *Byssochlamys*) can effect this conversion. It is certainly remarkable that the production of mannitol from glucose is so common, whilst there is no known case of the conversion by moulds of glucose into sorbitol, a change which is easy to effect by purely chemical reduction.

EXPERIMENTAL.

The culture medium used was the standard Czapek-Dox solution containing 5 % of glucose: water to 1000 cc.; glucose, 50 g.; NaNO_3 , 2 g.; KH_2PO_4 , 1 g.; KCl , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g. Five large-scale experiments have been carried out, in each case using 100 conical flasks, of 1-litre capacity, each flask containing 350 cc. of solution. The medium was sterilised by steaming for an hour on each of three consecutive days and was then sown with a spore suspension made from 6 cultures of *B. fulva* on wort-agar slopes, the cultures being at least 10 days old.

In the first experiment three of the flasks were incubated at 37°, three at 30° and the remainder at 24°. At 37° growth was exceedingly slow at first owing to the fact that the spores used for inoculation were completely wetted and sank to the bottom of the liquid, with the result that a gelatinous, submerged mycelium was slowly formed, and only when this was sufficiently extensive to reach the surface were normal aerial colonies produced. After this, growth was rapid, but at no stage did the solution give a precipitate on addition of acid. The results of analyses of the contents of flasks incubated at 30° and 24° are given in Table I. The column headed "On acidification" indicates the result

Table I.

Days incubation	30°			24°		
	p_{H}	% glucose by polarimeter	On acidification	p_{H}	% glucose by polarimeter	On acidification
18	4.5	3.87	Solution clear	4.7	4.33	Solution clear
32	4.5	1.61	Faint opalescence	4.8	2.26	Slight precipitate
57	4.5	Nil	Very slight precipitate	4.8	Nil	Considerable precipitate

obtained by making the filtered metabolism solution acid to Congo red by addition of HCl , when the appearance of the liquid is a rough indication of the amount of the specific product. The sugar is thus utilised more rapidly at 30° than at 24° (this is confirmed by the growth rate as judged visually), but less of the specific product is formed.

The contents of the remaining 91 flasks were worked up, as described below, for isolation of products at the end of 57 days' incubation.

In the second and subsequent experiments all the flasks were incubated at 24° until the solution had a rotation close to zero, the periods varying from 56 to 63 days. At the end of the incubation period the contents of the flasks were filtered, the mycelium was well squeezed out and washed with water, and

an average sample of the mixed filtrate and washings taken for analysis. To the main bulk of liquid was added, with vigorous stirring, sufficient concentrated HCl to make the liquid definitely acid to Congo red (100 cc. acid). A yellow flocculent precipitate formed at once and, after standing overnight, was filtered off, washed with water and dried *in vacuo* over H_2SO_4 (acid precipitate, see below). In no case did further addition of acid to the mother-liquor give any more precipitate.

In Exp. 1, the mother-liquor was neutralised to methyl red by 450 cc. of 2N NaOH and then evaporated to 800 cc. *in vacuo* at 45–50°. On cooling, the concentrate deposited a large amount of semi-crystalline solid and, on standing at 0°, a further quantity of solid separated in the form of long, colourless needles. A small amount of the latter was taken out, drained at the pump and cautiously washed with cold water till free from the brown mother-liquor. The crystals were perfectly colourless, dissolved readily in cold water, had m.p. 166–167° and, mixed with a sample of pure mannitol, melted at 166–167°. By recrystallisation from 66 % alcohol of the main bulk of solid matter the latter was shown to consist almost entirely of mannitol mixed with small amounts of inorganic salts.

In Exps. 2, 3 and 4 mannitol was estimated in the filtered metabolism solution, using the polarimetric method of Raistrick and Young [1931] (see Table III, column 5). The total volume of solution in each case was measured and the total production of mannitol from the 1750 g. of glucose originally present was calculated. In Exp. 4 the metabolism solution, after removal of the acid precipitate, was concentrated *in vacuo* (without previous neutralisation), the separated solid recrystallised from 66 % alcohol and the mother-liquor alternately concentrated and treated with 2 volumes of alcohol until no further mannitol could be obtained. The total recovery of mannitol, attained without any preliminary purification of the solution, was 80 % of the estimated amount.

Treatment of the acid precipitate. In Exp. 1 the dry solid was yellowish-brown in colour and weighed 18.1 g. It was extracted with ether in a Soxhlet apparatus until nothing further was dissolved. Crystals soon began to separate in the extraction flask and gradually a dirty green solid, slowly turning black, was deposited above the liquid level, the ether solution being reddish-brown. The extraction was stopped at intervals, the crystalline solid being filtered off, well washed with cold ether and dried in air. The total time of extraction was approximately 16 hours, and three solid fractions were obtained. A further quantity of solid was obtained by concentration of the combined mother-liquors, after which further evaporation gave only a thick brown syrup. The characteristics of the four solid fractions are given in Table II. Mixtures of any of the fractions

Table II.

Fraction	Colour	Weight g.	M.P.
a	Faint yellow	1.70	163°
b	Pale bluish grey	4.15	162–163°
c	Dark grey	1.70	162–163°
d	Faint yellow	0.48	163°

all melted at 162–163°, showing that all consisted essentially of the same substance. From the mycelium of the fungus a further quantity of identical material was obtained. The mycelium was dried *in vacuo* at a low temperature, finely powdered and extracted first with light petroleum, which rapidly removed a quantity of dark brown sticky material. Subsequent extraction with ether gave

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1.08 g. of almost colourless prisms, M.P. 162.5–163°, unchanged by mixing with fraction "a" (above).

The crude crystalline material was purified as follows. It was dissolved in benzene (approximately 6 cc. to every 1 g. of substance), boiled for some time to coagulate traces of the greenish pigment, the solution filtered hot and mixed with twice its volume of boiling ether. On cooling, colourless prisms, M.P. 163°, separated in almost quantitative yield.

The acid precipitates obtained in Exps. 2–5 had similar characteristics and were treated in the same way.

The yields of metabolic products, and other experimental details, are given in Table III.

Table III.

Experiment number	Incubation period days	Metabolism solution		Mannitol		Wt. of acid precipitate g.	Wt. of byssochlamic acid g.
		Volume litres	Rotation 2 dm. tube Hg ₅₄₆₁	Estimated g.	Isolated g.		
1	57	—	Nil	—	—	18.1	8.03
2	63	31.8	+ 0.23°	597	—	16.8	6.28
3	56	31.35	+ 0.025°	498	—	20.1	7.20
4	56	31.48	– 0.03°	460	366	19.8	6.13
5	59	—	– 0.06°	—	—	23.5	8.10

Characterisation of mannitol. All the mannitol isolated was in the form of colourless needles, M.P. 166°, and, mixed with an authentic sample of pure mannitol, had M.P. 166–167°. In a 2 dm. tube the rotation of a 2 % solution was $\alpha_{5461}^{18^\circ} - 0.02^\circ$, whilst a 2 % solution in a 6 % borax solution gave, in a 4 dm. tube, $\alpha_{5461}^{18^\circ} + 2.92^\circ$ and $\alpha_{5790}^{18^\circ} + 2.56^\circ$. The mean percentage of mannitol in the solution, calculated from the rotations using the tables of Raistrick and Young [1931], is 2.0.

Byssochlamic acid. Byssochlamic acid was obtained by crystallisation from a mixture of ether and benzene in the form of colourless prisms, M.P. 163°. Recrystallisation from alcohol gave crystals of the same form, M.P. 163.5°, and the melting-point was not raised by further recrystallisation from alcohol, or benzene and light petroleum. There is no obvious decomposition at the melting-point, and the melt is clear and colourless but does not resolidify on cooling, even when seeded. The pure substance is insoluble in water. It is slightly soluble in alcohol, the solution being faintly acid to litmus and giving no colour reaction with FeCl₃. Its solubilities, at room temperature, in various organic solvents, expressed as g. substance in 100 cc. of solvent are approximately: acetone, 63; ethyl acetate, 33; chloroform, 20; benzene, 7.5; ether, 0.33; alcohol, 0.3; light petroleum less than 0.1. By slow evaporation of solutions in acetone or benzene the substance may be obtained in beautiful rectangular prisms up to half-an-inch long. Byssochlamic acid is insoluble in cold dilute NaOH, but, on heating, dissolves slowly. It is reprecipitated unchanged by addition of mineral acid to the alkaline solution. It dissolves readily in warm concentrated HNO₃ and is recovered unchanged on evaporation of the solution. It is soluble in warm concentrated H₂SO₄ and is reprecipitated on addition of water. The H₂SO₄ solution darkens slowly when strongly heated.

Micro-analysis (Schoeller) gave: Sample 1: C, 65.11, 65.16 %; H, 6.12, 6.16 %; N, negative; OCH₃, negative; molecular weight, 310, 326. Sample 2: C, 65.15, 65.04 %; H, 6.10, 5.99 %. C₁₈H₂₀O₆ requires C, 65.03 %; H, 6.07 %; molecular weight, 332.

Estimations of molecular weight by depression of the freezing-point of benzene gave somewhat erratic results. In a 0.7 % freshly made solution, the observed molecular weight was 586. This fell gradually on standing to a final steady value of 322. In 1.3 % solution the final value was 352, and in a 2 % solution 407. The lowest values obtained indicate that the correct formula is $C_{18}H_{20}O_6$ and not $C_9H_{10}O_3$.

Titration with $N/10$ NaOH to phenolphthalein in aqueous solution can be carried out only if the temperature is maintained near the boiling-point. Even then solution proceeds very slowly but the end-point is quite sharp. 0.1973 g. substance required 23.62 cc. $N/10$ NaOH, whence the equivalent (E) is 83.5. In acetone solution titration to phenolphthalein proceeds smoothly in the cold and without lag. 0.2003 g. of substance, dissolved in 25 cc. of pure acetone, required a net amount, corrected for blank, of 24.01 cc. $N/10$ NaOH, whence $E=83.4$. $C_{18}H_{20}O_6$ titrating as a tetrabasic acid has $E=83.04$.

The specific rotation, using a 0.4 % solution in $CHCl_3$, is $[\alpha]_{5461}^{20} + 127^\circ$, $[\alpha]_{5790}^{20} + 108^\circ$.

The sodium salt is extremely soluble in water. Evaporation of the solution at room temperature *in vacuo* gives a syrup which slowly solidifies in micro-crystalline form. A 1 % solution of the sodium salt was used to test reactions with solutions of various metallic salts. Each test was made on 2 cc. of solution, the appropriate reagent being added drop by drop until no further reaction occurred, with the following results.

$AgNO_3$ gives a white, amorphous precipitate, readily soluble in dilute HNO_3 or NH_4OH , unchanged on standing for 24 hours but darkening gradually after several days or on prolonged heating at 100° . $BaCl_2$ gives a white precipitate separating slowly as rosettes of microscopic needles, and $FeCl_3$ a heavy, buff precipitate insoluble in excess of the reagent. Both normal and basic lead acetates give heavy, white amorphous precipitates. $CaCl_2$ gives no precipitate even on addition of four volumes of alcohol. Salts of Hg, Cu, Ni and U give no precipitates but, with the exception of Hg, give solutions which are much more intensely coloured than the reagents used.

A quantity of the silver salt was prepared by adding approximately 100 % excess of the theoretical amount of $AgNO_3$ to a neutral solution of the sodium salt, the white amorphous precipitate being filtered off, washed and dried at 100° . By ignition, Ag, 54.11, 54.10 %. Calculated for $C_{18}H_{20}O_8Ag$: Ag, 54.23 %.

An unsuccessful attempt was made to prepare the free acid $C_{18}H_{24}O_8$ by cautious acidification of the sodium salt. 0.5193 g. of byssochlamic acid was dissolved by heating with 62.5 cc. of $N/10$ NaOH. The cooled solution was covered with 200 cc. of ether, and 59.5 cc. of $N/10$ H_2SO_4 (approximately 5 % less than the theoretical amount) were added in 2 cc. portions, shaking vigorously between additions. The ether solution was evaporated at room temperature *in vacuo* and the white residue dried by exposure to air. The m.p. was 163° , with no visible change at any lower temperature. A sample heated to constant weight at 100° lost 1.1 %, the calculated loss, from $C_{18}H_{24}O_8$ changing to $C_{18}H_{20}O_6$, being 9.8 %.

Toxicity of byssochlamic acid. Toxicity tests have very kindly been carried out for us by Prof. Topley of the London School of Hygiene and Tropical Medicine. Twelve mice in three groups of four were injected with a solution of the readily soluble sodium salt. Three solutions in normal saline were made, such that the required amounts of substance were contained in 0.5 cc. The mice in the first group each received an intraperitoneal injection of 25 mg. of the sodium salt, and all were dead in 24 hours. The second group received 12.5 mg.

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and, of these, one died in 24 hours, two after 2 days and one after 3 days. The third group were injected with 6.25 mg.; one died in 2 days, two in 3 days and one in 6 days. The substance thus shows a definite, though low, toxicity to mice.

Further work is in progress with the object of elucidating the molecular structure of byssochlamic acid and will be reported in due course.

SUMMARY.

1. *Byssochlamys fulva* Olliver and Smith has been grown on Czapek-Dox solution at 24°, and it is shown that the metabolic product formed in greatest amount is mannitol. Yields equivalent to 30 % of the sugar consumed were obtained.

2. A new mould product, byssochlamic acid, $C_{18}H_{20}O_6$, M.P. 163.5°, has also been isolated from the metabolism solution, in yields of about 0.5 %. Byssochlamic acid is toxic to mice.

Our best thanks are tendered to Mr T. Rendle, of Messrs Chivers and Sons, Ltd., Histon, Cambridge, who supplied us with cultures of *Byssochlamys fulva*.

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CCXLVII. DIURNAL VARIATIONS IN THE BLOOD-SUGAR LEVEL OF THE LACTATING COW.

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(Received September 7th, 1933.)

LITERATURE relating to the bovine blood-sugar level shows that there is no concord of opinion as to the normal value. Among the earlier workers the average values recorded range from 50 mg. per 100 cc. [Awdejewa *et al.*, 1927] to 82 mg. per 100 cc. [Schwarz, 1928]. Hewitt [1930] reports values of 79-95 mg. per 100 cc. for dry cows and 50-63 mg. per 100 cc. for lactating cows, and quotes average values ranging from 41 to 80-120 mg. per 100 cc. from a number of other workers. Hodgson *et al.* [1932] give an average value of 53 mg. per 100 cc. for cattle over 2 years old, and Turk and Work [1933] give 51 mg. per 100 cc. as their average for lactating cows. While it is conceivable that part of this variability is due to the different methods of analysis employed, the question of the time of sampling must not be lost sight of as a possible factor.

The question of diurnal variation has so far received little attention. Richter [1928] could find no evidence of diurnal variation. Little *et al.* [1928], studied one cow, sampled hourly from 5 p.m. to 9 p.m. and again from 6 a.m. to 4 p.m. the following day, and obtained this sequence of figures: 56, 69, 62, 73 and 66 mg. per 100 cc. for the evening samples and 66, 76, 90, 90, —, 76, 83, 93, 78, 69 and 79 mg. per 100 cc. the next day. Hodgson *et al.* [1932] sampled cows, which were fed at 6 a.m. and 4 p.m., at 7 and 10 a.m. and 1, 3 and 5 p.m., and obtained blood-sugar values of 51.4, 49.0, 50.7, 52.9 and 50.5 mg. per 100 cc. at these hours. Allcroft and Strand [1933] reported higher blood-sugar levels in the evening than in the morning with starving sheep.

EXPERIMENTAL.

In the course of regular sampling for blood considerable fluctuations of blood-sugar level in the dairy cows were observed, notably when a large number of cows were bled on the same day and the time of sampling was extended. The general tendency for the later-drawn samples to exhibit lower levels of blood-sugar suggested that there might be a steady diurnal decrease about this time, which might be part of a diurnal rhythm in the level of this constituent. The following work was therefore undertaken to test the question, the Hagedorn-Jensen blood-sugar technique being employed [1920].

Exp. 1. One cow was sampled hourly from 6 a.m. till 11 a.m. and the blood-sugar values obtained were 68, 67, 65, 59, 51 and 42 mg. per 100 cc., showing a steady decrease which was more marked in its later stages.

Exp. 2. Two cows were sampled hourly from 6 a.m. till 3 p.m., their values for blood-sugar being given in Table I.

Table I.

Time	6 a.m.	7 a.m.	8 a.m.	9 a.m.	10 a.m.	11 a.m.	Noon	1 p.m.	2 p.m.	3 p.m.
Cow 9	78	73	70	65	65	58	55	48	55	64
„ 18	64	54	54	53	68	65	—	42	58	68

These two preliminary tests were sufficiently suggestive to warrant fuller investigation.

Exp. 3. Four cows, Nos. 9, 10, 17 and 18, near the end of their lactation period, were used. Samples were taken hourly from 3 a.m. till 11 p.m. and again, after a period of rest for the cows, from 3 a.m. to 7 a.m. the next morning. Cows 9 and 10 were sampled at the odd hours and cows 17 and 18 at the even hours, so that each cow was sampled every 2 hours. From the data obtained a composite curve was constructed taking at each half-hour the average of the four cows, two half an hour before and the other two half an hour after; *i.e.* 7.30 represents the average of the four bloods taken at 7 and 8 o'clock. This curve is shown in Fig. 1 and exhibits a sharp decline from 7.30 a.m. to about 11.30 a.m. followed by a small rise to 3.30 p.m. and subsequent decrease. It was thought that the continual disturbance of the cows might account for the failure of the blood-sugar level to return to its high value in the early morning, and so for subsequent work the technique of sampling was modified.

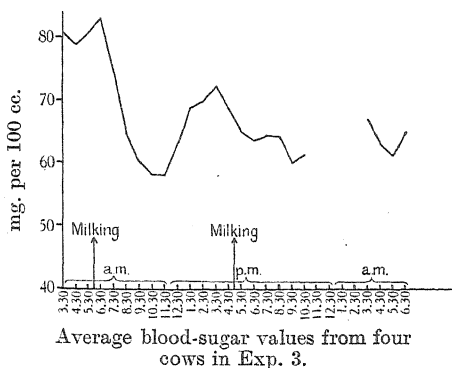


Fig. 1.

Exp. 4. To minimise the risk of continued disturbance it was decided to extend the experiment over 24 days, sampling only once a day, an hour later

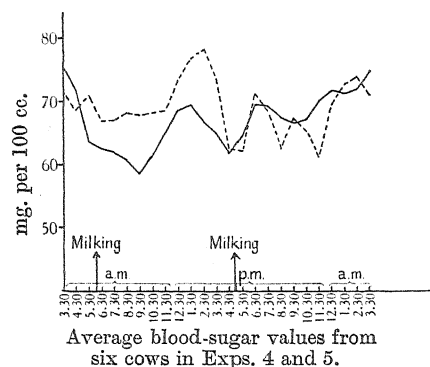


Fig. 2.

---- Dry cows [Exp. 4].
 — Lactating cows [Exp. 5].

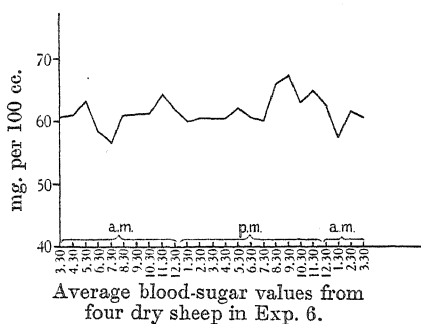


Fig. 3.

each day. Previous work (unpublished) had shown that over a period of a week variations up to 16 % of the blood-sugar value were likely to occur when samples were drawn at the same hour each day. Six cows were used and divided into two

lots of three, one lot being bled at the odd hours and the other lot at the even hours, so that each lot of cows was only bled once in two days. The cows were dry at the time of this experiment. A composite curve, constructed as was Fig. 1, is shown by the broken line in Fig. 2.

Exp. 5. This was a repetition of Exp. 4, using the same animals in the same groups, when the cows were in full lactation, some 7 to 10 weeks after calving. Their composite curve is given by the continuous line in Fig. 2.

Exp. 6. To act as a check on the dry cows' curves four dry sheep, two black-faced Highland wethers and two non-pregnant half-bred ewes, were used and sampled hourly over an extended period. Their composite curve is given in Fig. 3.

DISCUSSION.

From the curves given in Figs. 1, 2 and 3 it is safe to conclude that, while there is no apparent diurnal rhythm in the blood-sugar level of dry cows and dry sheep, there is some evidence of a decided trend of diurnal changes in the blood-sugar level of lactating cows. In Fig. 1 the curve falls rapidly from 7.30 a.m. till 11.30 a.m. after which it rises slightly and falls again; its continued low level next morning was suspected to be due to the fact that the animals had been disturbed hourly over the early part of the night at a time when they would normally be quiescent. That this theory was in part at least correct is suggested by the adequate recovery obtained in Exp. 4, Fig. 2, when all possible precautions were taken to avoid unnecessary disturbance of the animals. This latter curve does not exhibit the same extreme fluctuations as does the curve in Fig. 1, nor do the low peaks of the two curves exactly coincide for time of day, but the curves are so strongly similar in character as to suggest the existence of a definite diurnal rhythm in the level of blood-sugar. The fact that no such rhythm is apparent in the curves for dry cows or dry sheep would seem to imply that this rhythm is associated with the state of lactation. This suggestion is in keeping with the work of Filipovic [1931], who observed that the rate of secretion of milk increased rapidly to a maximum about 4 to 5 hours after milking and thereafter slowed down again, and of Carlens and Krestownikof [1927], who noted that the level of blood-sugar was high before milking and fell during milking, unless the animal was unduly disturbed by the sampling.

In all the work reported here the schedule of feeding hours was the same and, in the case of lactating animals, the hours of milking were the same.

It is not the purpose of this communication to discuss or theorise over the possible physiological causes of the variations found but rather to emphasise the fact that, especially when dealing with lactating animals, it is highly important to keep the hour of sampling constant when comparable blood-sugar values are required. Further work, designed to discover the factor or factors causing the variation, would be of assistance in interpreting results obtained from various sources.

I wish to acknowledge my indebtedness to Mr W. Thomson for invaluable assistance with the sampling and to Mr W. Godden for his interest in this work.

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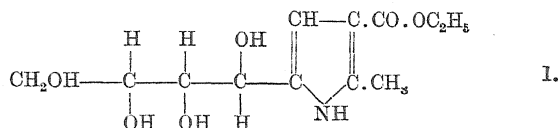
CCXLVIII. A COLORIMETRIC METHOD FOR THE DETERMINATION OF GLUCOSAMINE AND CHONDROSAMINE.

By LESLIE ALDERMAN ELSON¹
AND WALTER THOMAS JAMES MORGAN.

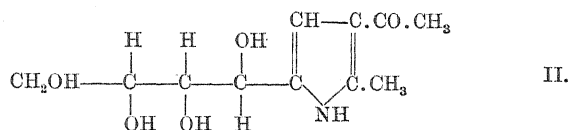
From the Serum Department, The Lister Institute, Elstree, Herts.

(Received October 27th, 1933.)

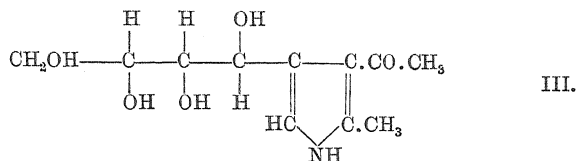
A COLORIMETRIC method for the determination of glucosamine has recently been described by Zuckerkandl and Messiner-Klebermass [1931]. According to their procedure the test-solution, which should contain between 1 and 4 mg. glucosamine hydrochloride, is evaporated to dryness and treated with a freshly prepared solution of sodium methoxide. The free glucosamine base is then cautiously acetylated with acetic anhydride to yield the *N*-monoacetyl derivative which can be estimated by means of the intense reddish-purple colour that develops when, after a preliminary treatment with dilute alkali, *p*-dimethylamino-benzaldehyde in acid solution (Ehrlich's reagent) is added. We have found this method unsatisfactory. The acetylation of glucosamine which yields the *N*-monoacetyl derivative, under the conditions described by Zuckerkandl and Messiner-Klebermass does not appear to be quantitative and, moreover, rapid fading of the colour that develops on the addition of the *p*-dimethylamino-benzaldehyde reagent increases the difficulty of colorimetric estimation. That Zuckerkandl and Messiner-Klebermass were aware of the difficulties involved in the use of the method for quantitative purposes is evident from their description of the reaction. They state that the error of estimation is, at the most, 6 %.



Ethyl ester of 2-methyl-5-tetrahydroxybutylpyrrole-3-carboxylic acid.



3-Acetyl-2-methyl-5-tetrahydroxybutylpyrrole.



3-Acetyl-2-methyl-4-tetrahydroxybutylpyrrole.

¹ Jenner Research Scholar.

A colorimetric method for the determination of glucosamine has therefore been elaborated which avoids the process of evaporation to dryness and subsequent conversion of the glucosamine into its *N*-monoacetyl derivative and which gives rise to very stable coloured solutions. The method depends upon the colour which develops when pyrroles are condensed with *p*-dimethylaminobenzaldehyde. The conversion of glucosamine into the pyrrole derivatives (I) and (II), by the action of ethyl acetoacetate and acetylacetone respectively has been described by Pauly and Ludwig [1922]. When glucosamine hydrochloride is boiled in alkaline solution with either ethyl acetoacetate or acetylacetone the resulting solutions, on treatment with Ehrlich's reagent in the presence of alcohol, develop a stable red colour. The colour obtained with the condensation product of acetylacetone (II) was found to be the more intense and for this reason was selected as a basis for the colorimetric determination described below.

EXPERIMENTAL.

Reagents. (1) *Acetylacetone solution.* The reagent is made by dissolving 1 cc. of acetylacetone in 50 cc. of 0.5 *N* sodium carbonate solution; the acetylacetone dissolves readily on shaking. The reagent should be kept in an ice-chest when not in use and should be prepared fresh every 4 or 5 days.

(2) *p-Dimethylaminobenzaldehyde reagent.* *p*-Dimethylaminobenzaldehyde (0.8 g.) which has been twice recrystallised from dilute alcohol is dissolved in alcohol (30 cc.) and concentrated hydrochloric acid (30 cc.) added. The reagent possesses a pale yellow colour and keeps indefinitely.

(3) *Glucosamine hydrochloride standard.* An aqueous solution of glucosamine hydrochloride saturated with chloroform and containing 10 mg. of the hydrochloride in 1 cc. is prepared. From this solution suitable dilutions can be made when required. The standard solution should be kept between 0 and 4°.

Procedure. The determination is best carried out in test-tubes graduated at a volume of 10 cc. The solution to be estimated, which should contain between 0.5 and 3.0 mg. of glucosamine hydrochloride, is pipetted into the tubes and the acetylacetone reagent (1 cc.) added from a pipette; the sides of the tubes are then washed down with 1 cc. of water. At the same time standard solutions of glucosamine hydrochloride are measured out and treated in the same manner. The tubes are heated for 15 minutes in a boiling water-bath in which the level of the water is kept just above the level of the liquid in the tubes. The upper portion of the test-tubes should project out of the water-bath in order to avoid any serious loss of acetylacetone by evaporation. After heating, the tubes are cooled and alcohol is added to within about 2 cc. of the 10 cc. graduation mark. During the addition of alcohol a precipitate frequently forms but it is rapidly dissolved on the subsequent addition of the Ehrlich reagent (1 cc.); alcohol is then added to make the volume up to 10 cc. The colours develop quickly and reach their full intensity in 15–20 minutes. During this time there is a slow evolution of carbon dioxide which renders colorimetric comparison difficult, and it has been found convenient to compare the colours after the solutions have stood for at least 30 minutes at room temperature. When compared with a stable artificial colour standard the red colour which develops shows no fading over a period of several hours.

The colorimetric comparison is reliable only when the intensities of the tints of the unknown solution and the standard solution are approximately the same. It has been found that a comparison of colours is difficult if the glucosamine contents of the solutions differ by more than 25 %. The accuracy of the method

is readily ascertained from the results shown in Table I where it will be seen that the error of estimation is less than 5 % if the amount of glucosamine hydrochloride is within the range 0.75-3.0 mg. and the colour intensity of the standard glucosamine hydrochloride solution does not differ by more than 25 % from that of the unknown.

Table I.

Glucosamine hydrochloride				Glucosamine hydrochloride			
Standard solution mg.	Present mg.	Found mg.	Percentage error	Standard solution mg.	Present mg.	Found mg.	Percentage error
0.25	0.50	0.475	- 5.0	2.00	1.80	1.84	+ 2.2
"	"	0.530	+ 6.0	"	"	1.80	0.0
0.75	"	0.509	+ 1.8	"	2.20	2.17	- 1.3
"	"	0.520	+ 4.0	"	"	2.19	- 0.5
"	1.00	1.02	+ 2.0	"	2.50	2.50	0.0
"	"	1.00	0.0	"	"	2.45	- 2.0
1.00	0.80	0.830	+ 3.7	2.50	2.25	2.24	- 0.5
"	"	0.827	+ 3.3	"	"	2.21	- 1.8
"	1.20	1.21	+ 0.8	"	2.75	2.80	+ 1.8
"	"	1.22	+ 1.6	"	"	2.78	+ 1.1
"	1.40	1.35	- 3.5	"	3.00	3.07	+ 2.3
"	"	1.39	- 0.7	"	"	2.02	+ 0.7
1.50	1.20	1.22	+ 1.6	3.00	2.50	2.50	0.0
"	"	1.23	+ 2.4	"	"	2.54	+ 1.6
"	1.80	1.80	0.0	"	3.50	3.40	- 3.0
"	"	1.79	- 0.6	"	"	3.32	- 5.1
				"	4.00	3.75	- 6.2

This method can also be used for the determination of chondrosamine. Equal amounts of glucosamine hydrochloride and chondrosamine hydrochloride give rise to colours identical in tint and intensity. A few of the results obtained by using standard solutions of chondrosamine hydrochloride are given in Table II; it will be seen that the limits of accuracy of the determination as given for glucosamine hydrochloride also hold for chondrosamine hydrochloride.

Table II.

Chondrosamine hydrochloride			
Standard solution mg.	Present mg.	Found mg.	Percentage error
1.50	1.00	0.992	- 0.8
1.50	1.00	1.00	0.0
1.50	2.00	2.00	0.0
2.00	2.50	2.42	- 3.2

The influence of foreign substances.

In order to ascertain whether the presence of sugars has any influence upon the determination of glucosamine a number of estimations have been made in which glucose, galactose, fructose or arabinose was added to the glucosamine solution before estimation. The results of these tests are given in Table III and it will be seen that in no case had the added substance any appreciable influence upon the accuracy of the estimation. Similarly the presence of glycine, alanine or histidine does not interfere with the determination.

For obvious reasons the method cannot be used when certain pyrrole or indole derivatives are present; these substances, however, can be readily detected since they will condense with *p*-dimethylaminobenzaldehyde in acid

Table III. *The influence of foreign substances upon the accuracy of the estimation.*

Substance added	mg.	Glucosamine hydrochloride		Percentage error
		Present mg.	Found mg.	
Glucose	2.5	2.0	1.97	- 1.5
	5.0	"	2.02	+ 1.0
Galactose	5.0	"	2.03	+ 1.5
	10.0	"	1.99	- 0.5
Fructose	2.5	"	2.02	+ 1.0
	5.0	"	2.01	+ 0.5
Arabinose	2.5	"	1.98	- 1.0
	5.0	"	2.00	0.0
Glycine	2.5	"	2.00	0.0
	5.0	"	1.97	- 1.5
Alanine	2.0	"	2.03	+ 1.5
	2.0	"	1.98	- 1.0
Histidine	1.0	"	2.00	0.0
	1.0	"	2.03	+ 1.5
Tryptophan	1.0	"	2.04	+ 2.0
	1.0	"	2.03	+ 1.5

solution to yield coloured solutions without previous heating with the acetyl-acetone reagent. The presence of tryptophan does not affect the accuracy of the estimation because the colour which this amino-acid is known to give with Ehrlich's reagent does not develop in the presence of hydrochloric acid of the strength used in the determination; the tryptophan reaction requires a much greater concentration of acid. *N*-Acetylglucosamine and *N*-acetylchondrosamine, if present, will also yield a reddish-purple coloration with Ehrlich's reagent, but only after heating in alkaline solution. The coloration produced with these two compounds, however, is not due to the formation of pyrrole derivatives, but to the elimination of the elements of water, followed by ring-closure with the formation of a disubstituted oxazole [Elson and Morgan, 1933, 2], which condenses with *p*-dimethylaminobenzaldehyde to yield an intense reddish-purple coloured solution [Elson and Morgan, 1933, 1].

Although 1-aminoglucose is not known to occur naturally and is, therefore, unlikely to be present in the acid hydrolysis products of either glucoproteins or nitrogen-containing polysaccharides it is of interest that this aminohexose, prepared according to the method of Ling and Nanji [1922] condenses readily with acetylacetone, under the conditions described above for the estimation of glucosamine and chondrosamine, to yield a coloured solution almost identical in tint and intensity with that produced by equal quantities of these compounds. In this case the acetylacetone presumably combines with 1-amino-glucose to give the compound (III) which on treatment with Ehrlich's reagent gives the usual pyrrole colour.

The influence of the concentration of hydrochloric acid on the rate of development of the colour¹.

Four colorimetric determinations were made, using 1.0 mg. of glucosamine hydrochloride, in which the concentration of hydrochloric acid present in the final solution was the only factor that was varied. The results, as shown in

¹ (Note added November 21st.) In the presence of considerably higher concentrations of hydrochloric acid (> 2*N*) it has been found that many carbohydrates and amino-acids, after being heated with acetylacetone in alkaline solution, react with *p*-dimethylaminobenzaldehyde and give rise to red-coloured solutions.

Table IV. *The influence of concentration of hydrochloric acid on the rate and intensity of colour development.*

Final concentration of hydrochloric acid %	Time of colour development (minutes)						
	5	10	15	20	30	40	60
	Colorimeter readings (mm.)						
2.5	34.5	32.5	30.0	28.7	26.0	25.3	24.5
5.0	27.0	24.9	24.6	24.3	24.3	24.4	24.3
10.0	24.7	24.8	25.0	24.8	24.9	25.2	25.4

Table IV, were obtained by matching the colours which developed in the presence of the various hydrochloric acid concentrations, after definite intervals of time, against a stable artificial colour standard. It will be noted that in the presence of 5 % hydrochloric acid the colour develops rapidly and is constant in intensity after 20 minutes. Moreover, this concentration of hydrochloric acid gives rise to the greatest depth of colour and has therefore been selected as the most suitable concentration of acid for use in the preparation of the *p*-dimethylaminobenzaldehyde reagent.

The influence of the time of heating on the colour development.

Two tests were carried out in the same manner as those already described except that the glucosamine hydrochloride solutions were heated with the acetylacetone reagent for different periods of time. It will be observed from the colorimeter readings given in Table V that the maximum colour intensity

Table V. *The influence of the time of heating with the acetylacetone reagent on the subsequent colour development.*

Time of heating at 100° (minutes)	Colorimeter readings (mm.)	
	Exp. No. 1	Exp. No. 2
10	25.0	24.2
15	18.1	17.8
20	23.0	27.2

develops after the solutions have been heated for 15 minutes. It has been found that the colours obtained after heating solutions of glucosamine hydrochloride with the acetylacetone reagent for 20 minutes or longer, are of a slightly different tint from those obtained with solutions which have been heated for 10 or 15 minutes.

SUMMARY.

A method is described for the colorimetric estimation of glucosamine and chondrosamine. By heating these substances in alkaline solution with acetylacetone they can be converted into pyrrole derivatives which on treatment with *p*-dimethylaminobenzaldehyde give rise to very stable red-coloured solutions. Within limits the method gives a good degree of proportionality between the hexosamine content and the colour intensity.

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CCXLIX. A NEW MICRO-QUINHYDRONE ELECTRODE.

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(Received October 30th, 1933.)

INTRODUCTION.

THE micro-determination of the hydrogen ion concentration of a fluid is indispensable for some biological studies, and the quinhydrone electrode is well adapted for this purpose. Cullen and Büllmann [1925], and also Lang [1930], devised capillary electrodes which are however not suitable for a single drop. Gesell's bakelite tube electrode [1928] enables one to carry out four or five determinations with 0.2 cc., but its construction and manipulation are not very simple. I have devised a new type of micro-quinhydrone electrode at Professor Shoji's suggestion, which is applicable even to 0.01 cc. of a liquid. Its construction is quite simple. A thin gold tube, resembling a wide injection syringe needle, about 1 mm. in bore, serves as the electrode. The entire outer surface and the terminal third of each end of the inner surface are well covered with a suitable insulating varnish. One drop of the fluid to be examined is mixed with quinhydrone powder and the mixture sucked into the tube. Then the tip of the tube is thrust into KCl-agar-agar, which is connected, on the other side, with a calomel electrode or a standard quinhydrone electrode. The potential of the gold tube against the standard electrode is then measured in the usual way. The gold electrode tube, attached to a vulcanised rubber nipple of an ordinary injection needle, is illustrated in Fig. 1, which will suffice to explain its construction. Some remarks are necessary however on the preparation and use of the apparatus.

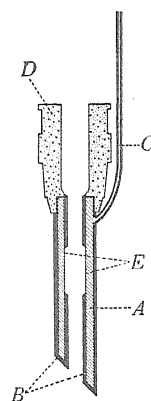


Fig. 1.

- A Gold tube electrode;
- B Insulating varnish;
- C Leading wire;
- D Hard rubber nipple;
- E Gilded portion of tube.

Preparation of the electrode.

It is not necessary to make the electrode tube of pure gold; 20 ct. or 18 ct. gold will suffice. In every case, however, even with a tube of 24 ct. gold, the inner surface of the tube, which is utilised as the metal electrode, must be perfectly gilded by electro-deposition. The gilding current should not be too large. I used a resistance of about 20,000 ohms in the circuit of one accumulator, and allowed deposition to proceed for 0.5 or 1 hour, the gold chloride solution inside the tube being renewed every five minutes, and thus always obtained a

good result. After the inside of the tube is gilded, it must not be heated or damaged mechanically, otherwise the base metal contained in the gold (a trace of which exists even in 24 ct. gold) comes into contact with the fluid and causes gross inaccuracies. For this reason the leading wire must be soldered to the electrode before it is gilded. The electrode once gilded perfectly may be used for a long time, but it is necessary frequently to check it with a standard solution; when it is found to have gone wrong, regilding will restore it again to good condition. A platinum tube was also tried but offered no advantages.

In order to prevent the electrode surface from being touched by the fluid unsaturated with quinhydrone, or any other fluid, the entire outer surface, and also preferably the terminal third of each end of the inner surface of the tube, as well as the leading wire, must be insulated completely. I found "Bakelite varnish No. 2," furnished by Sankyo and Co., Tokyo, quite satisfactory for this purpose, but of course any other insulating varnish will do. The hardening of the varnish requires a high temperature and must therefore be done before gilding. Neither paraffin nor enamel is suitable, as both these substances often crack easily.

Use of the electrode.

The actual estimation is carried out as follows. Quinhydrone powder, ground very finely, is put on a paraffined watch-glass. A small drop of the fluid is then put on the powder and is rolled quickly by moving the glass, so that the drop is well covered with the powder. Then the drop is sucked into the gold tube which is conveniently attached to a blind rubber tubing or the hard rubber nipple of an ordinary injection needle. The drop must be covered sufficiently with powder, otherwise the fluid may not be saturated with quinhydrone and an incorrect result may be obtained. Care must be taken that the amount of powder is not so excessive as to soak up the drop of fluid, but just enough to be sucked into the tube with the fluid.

If it is desired to avoid exposure to air a suitable amount of quinhydrone powder is put in a small hollow made at the bottom of a short test-tube and covered with liquid paraffin. The fluid is then introduced below the paraffin by means of a fine injection syringe and is mixed with the powder, after which it is sucked into the electrode tube. If paraffin is sucked into the electrode tube with the fluid, it does not harm the measurement unless the entire surface of the electrode be covered with paraffin. If this occurs no current passes through, and the fault can be easily detected.

Checking the results obtained by this method.

The method was checked by comparing its results with those obtained by the usual hydrogen-gas electrode method. This was done with McIlvain's buffer solutions, urine, aqueous humour, cerebrospinal fluid, blood-serum and also with whole blood. Results were quite satisfactory, except in the case of whole blood, so long as the value of the first half or one minute was taken. Later the value showed a deviation, which was very marked, especially with blood-serum. This is a defect not only of the present method, but of the quinhydrone method in general, as has already been discussed by many investigators.

In these estimations it is advisable to carry out three or four parallel determinations at the same time. Occasionally one electrode may give a value somewhat different from the others. Such a faulty electrode must be discarded. The table on the following page will serve as an example.

In this table the figures show that electrode No. 4 must be discarded.

Material	Temp. (°)	p_H by H-gas electrode	p_H by micro-quinhydrone method			
			No. 1	No. 2	No. 3	No. 4
McIlvain's buffer	18	7300	7303	7296	7300	7284
Blood-serum (horse)	18	7723	7722	7717	7722	7681
Blood-serum (horse)	37	7353	7351	7369	—	7319
Aqueous humour (rabbit)	18	7610	7614	7608	7617	—
Cerebrospinal fluid (rabbit)	18	7445	7466	7454	7464	—

If the p_H of a body fluid containing CO_2 , such, for instance, as blood-serum, is determined by this method without paraffin protection, CO_2 may be lost while the drop is rolled on the quinhydrone powder. I confirmed practically, however, that this did not occur, since the serum gave the same value with or without paraffin protection and with the H-gas electrode, using a large amount of the fluid.

When this method is applied to whole blood, it often gives a wrong (usually too alkaline) value, for which reason it is advisable to use plasma. The following procedure is convenient for determining the p_H of human blood. The finger-tip, or any other suitable place, is stabbed, and the wound is covered with liquid paraffin, under which the blood oozes out. About 0.3 cc. of the blood is sucked into a small syringe containing about 0.03 cc. of a 2.3 % potassium oxalate solution, whose p_H is adjusted to 7.4. The sample is well mixed and then centrifuged under liquid paraffin. The plasma thus separated is treated as described above.

I tried to utilise this method also for estimating the tissue fluid of a living animal. For this purpose a fine electrode needle was prepared. Quinhydrone powder, dry or slightly wet, was put inside, and the needle was introduced into the skin to reach the subcutaneous space. The tissue fluid should then come into the tube. Another electrode was filled with the saline solution and placed close to the former, and served as the connection with the calomel electrode. The results were always very uncertain. Even when such an electrode was immersed in a standard solution the result was still uncertain. This is probably due to the fact that the fluid entering the tube is not perfectly saturated with quinhydrone.

SUMMARY.

A minute drop of a fluid is mixed with fine quinhydrone powder and is sucked into a fine gold tube, which serves as the metal electrode. Then the p_H of the fluid is measured by the ordinary potentiometric method. The outer surface, and also preferably both ends of the inner surface of the tube, as well as the leading wire, should be insulated perfectly, lest they come into contact with the fluid unsaturated with quinhydrone. The inner electrode surface must be gilded perfectly.

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CCL. THE EFFECT OF NARCOTIC GASES ON BRAIN OXIDATIONS.

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(Received October 31st, 1933.)

In a previous paper [Bülow and Holmes, 1932], published from the Pharmacological Laboratory, Cambridge, we dealt with the effect of narcotics on the oxygen uptake of the brain tissue. We were unable to observe any decrease in the respiration of brain tissue *in vitro* in the presence of ethylene, propylene or nitrous oxide, gases of known anaesthetic power. A recent paper of Quastel and Wheatley [1932] has, however, made it desirable to carry out a fresh investigation of the subject.

With fresh tissue, Quastel's experiments are in full agreement with our own, in that he observes no difference in the oxygen uptake of chopped brain tissue, incubated in presence of a mixture of nitrogen and oxygen, or of narcotic gas and oxygen in the same proportions, but he thinks that this agreement is misleading, the real inhibition of oxidation being hidden by the greater solubility of narcotic gases in the cell-lipoids. If he uses tissue which has been allowed to become greatly depleted of its oxidisable material by aeration for 3 hours, and then adds glucose, he obtains in the following 2 hours a distinct inhibitory effect of the narcotics, the cell-lipoids now being saturated with the gas.

On theoretical grounds alone we do not think that Quastel's criticism is valid. Brain tissue contains nearly 8 % lipoids. The solubility of acetylene in oil as determined by Meyer *et al.* [1920] is 1.8 cc. in 1 cc. solvent, so that 43 mm.³ of acetylene may be absorbed by 0.3 g. brain, and so disappear. In an experiment lasting 3 hours the inhibition which can be masked by the absorption of the narcotic gas is thus quite small, and indeed falls well within the limits of experimental error of the method. Apart from this, the manometers are evacuated and filled with the gas mixture three times. After immersion in the thermostat, 15 minutes are allowed for the attainment of temperature equilibrium, before the taps are closed. In all probability, therefore, the lipoids are already saturated with gas before readings are commenced. Experiment confirmed these conclusions. The presence of 0.03 cc. olive oil caused no effect on the gas pressure in the Barcroft vessels, filled with propylene, nor did any change appear during 3 hours' shaking.

It seems possible to explain Quastel's results on the following lines. The oxygen uptake of the cell may be due at first to systems not affected by narcotics, and it may be necessary for the tissue to suffer material damage if it is to show the effect of narcotics on certain specific processes. Quastel removes the material, the oxidation of which is not affected by narcotics, by incubating the brain for 3 hours. We endeavoured to obtain the same effect by washing the tissue several times with distilled water.

As the experiment shows, washing brain tissue three times with distilled water diminishes the velocity of the oxygen uptake as much as did the process

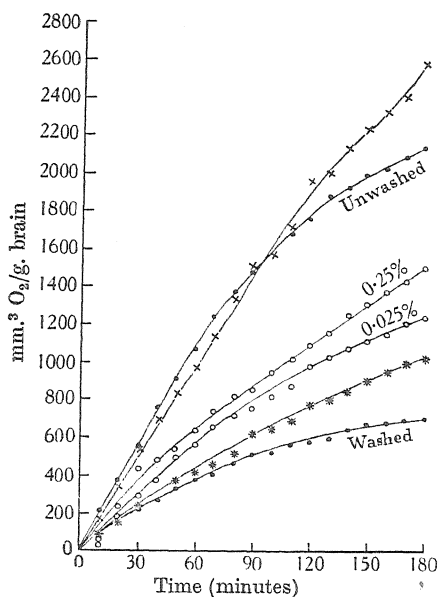


Fig. 1.

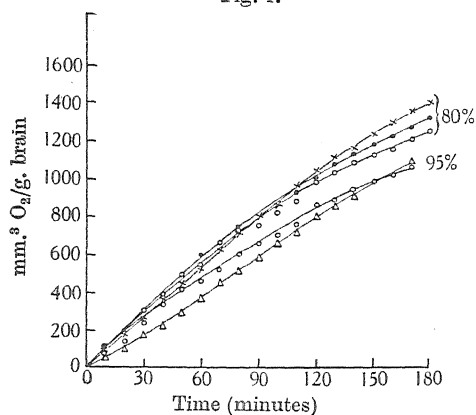


Fig. 2.

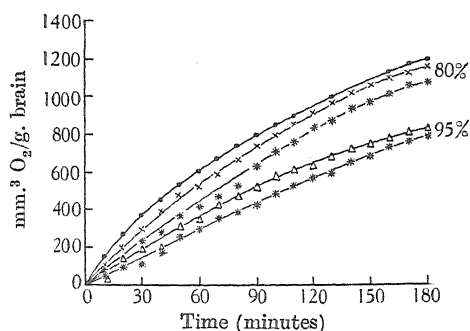


Fig. 3.

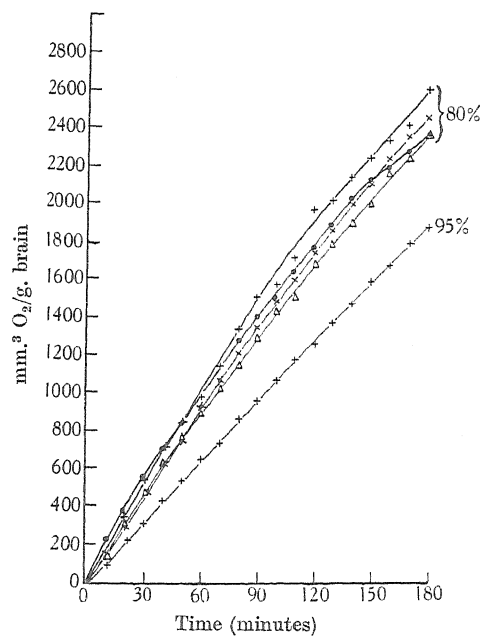


Fig. 4.

Fig. 1. O_2 uptake of brainalone $\bullet-\bullet$;Na lactate $*-*$;washed with added glucose $\circ-\circ$;washed with added Na succinate $\times-\times$.Fig. 2. O_2 uptake of washed brain with added glucose in the presence ofethylene 95% $\triangle-\triangle$;acetylene 80% $\times-\times$;propylene 80% $\bullet-\bullet$;nitrogen 80%, 95% $\circ-\circ$.Fig. 3. O_2 uptake of washed brain with added lactate in the presence ofethylene 95% $\triangle-\triangle$;acetylene 80% $\times-\times$;propylene 80% $\bullet-\bullet$;nitrogen 80%, 95% $*-*$.Fig. 4. O_2 uptake of washed brain and added succinate in the presence ofethylene 95% $\triangle-\triangle$;acetylene 80% $\times-\times$;propylene 80% $\bullet-\bullet$;nitrogen 80%, 95% $+ - +$.

of incubation in Quastel's experiments. The addition of glucose increases it distinctly (a solution that contains 0.025 % glucose gives nearly as great an effect as a solution of 0.25 %). The oxidation due to lactate is as great as that due to glucose, the effect of succinate being much more powerful (Fig. 1). These results, too, are in full agreement with Quastel's. But if the Barcroft vessels are now filled up with narcotic gas mixtures instead of air, and the oxygen uptake is compared with that which occurs in the presence of nitrogen-oxygen mixtures of the same oxygen content, there is no inhibition to be seen (Figs. 2, 3 and 4). This confirms our previous experiments and is in contradiction to Quastel's. The narcotics do not interfere with the oxidation either of glucose or of sodium lactate or sodium succinate. The last result, which is in agreement with Quastel's, seems to us of some general importance, and is difficult to bring into line with Keilin's studies [1925]. These indicate that the oxygen transference in the cell is inhibited by HCN, the activation of hydrogen by narcotics. The succinic acid dehydrase system is a typical dehydrase system, which has been much studied, and we should therefore expect it to be inhibited by these gases. As the experiments show, there is no such inhibition. On the contrary, in the experiments with ethylene the narcotic seems to have rather a stimulating effect. The uptake in a mixture of 95 % ethylene + 5 % oxygen is as large as that in 80 % acetylene or propylene and 20 % oxygen, and distinctly greater than that in 95 % nitrogen and 5 % oxygen.

EXPERIMENTAL.

0.3 g. of chopped mouse brain (the brains of 3 animals were usually mixed together) was suspended in about 10 cc. distilled water and centrifuged. The supernatant fluid was rejected. The process was repeated three times. The tissue was then placed in the right-hand vessels of Barcroft differential manometers which contained 3 cc. of Ringer solution, buffered with phosphate (4 parts of Ringer solution: 1 part of 0.2 M KH_2PO_4 solution) and brought to p_H 7.4. The left-hand vessels were filled in the same way, but without tissue. The filling with the gas was done by evacuating three times, and allowing the required mixture to run in. The CO_2 formed during the experiment was absorbed by 5 % KOH. All the gases were prepared in the laboratory, ethylene and propylene by treatment of ethyl and propyl alcohols with hot, water-free phosphoric acid, and acetylene from calcium carbide, the gas being washed with acidified $CuNO_3$ solution, dilute chromic acid, a mixture of $Ca(OCl)_2$ and CaO , and $NaOH$. The compositions of the gas mixtures employed were the same as in our previous experiments [1932], as they had been tested and proved to be narcotic for the mouse. (Quastel uses some lower concentrations.) The experiments were done at 36–38°, the apparatus being shaken for 15 minutes before closing the taps, and the readings taken at 10 minute intervals. The values are worked out for normal temperature and pressure. Each experiment was repeated at least four times, often more frequently. The results agreed very well.

As these experiments showed that with washed brain and added substrate narcotics caused no decrease in the oxygen uptake, we looked for another explanation of Quastel's results. An irreversible disturbance of the brain cells, due to a prolonged action of the narcotic, seemed to be a possibility.

To test this hypothesis we repeated Quastel's experiments, using his own method. Fresh brain tissue was shaken in Ringer solution, without adding substrate, and in the presence of narcotics. Controls contained a mixture of nitrogen and oxygen of the same oxygen content, and the same quantity of tissue. After 3 hours, when the rate of oxygen uptake had fallen distinctly, glucose was added and the apparatus refilled with the same gas mixture. In the following 2 hours we could confirm Quastel's observations. The oxygen uptake in this second period was decreased in the presence of acetylene and oxygen, compared with that in nitrogen and oxygen. But this effect was only given by acetylene. Ethylene and propylene did not act in the same way. With these gases the

Table I.

	Gas mixture $N_2 : O_2 = 4 : 1$	Gas mixture $CH \equiv CH : O_2 = 4 : 1$	Inhibition %
O_2 uptake in mm. ³ of 1 g. tissue alone in the 4th and 5th hours	304	262	14
Extra O_2 in presence of glucose (0.025 %)	561	125	78
	Gas O_2 previously $N_2 : O_2$	Gas O_2 previously $CH \equiv CH : O_2$	
O_2 uptake in mm. ³ of 1 g. tissue alone in the 6th and 7th hours	388	278	28
Extra O_2 in presence of glucose (0.025 %)	602	247	59
	Gas mixture $N_2 : O_2 = 1 : 1$	Gas mixture $CH \equiv CH : O_2 = 1 : 1$	
O_2 uptake in mm. ³ of 1 g. tissue alone in the 4th and 5th hours	297	280	—
Extra O_2 in presence of glucose (0.025 %)	398	217	45
	Gas O_2 previously $N_2 : O_2$	Gas O_2 previously $CH \equiv CH : O_2$	
O_2 uptake in mm. ³ of 1 g. tissue alone in the 6th and 7th hours	235	108	54
Extra O_2 in presence of glucose (0.025 %)	318	142	55
	Gas mixture $N_2 : O_2 = 4 : 1$	Gas mixture $CH_3CH_2=CH_2 : O_2 = 4 : 1$	
O_2 uptake in mm. ³ of 1 g. tissue alone in the 4th and 5th hours	255	390	—
Extra O_2 in presence of glucose (0.025 %)	820	790	—
	Gas O_2 previously $N_2 : O_2$	Gas O_2 previously $CH_3CH_2=CH_2 : O_2$	
O_2 uptake in mm. ³ of 1 g. tissue alone in the 6th and 7th hours	170	187	—
Extra O_2 in presence of glucose (0.025 %)	720	618	14
	Gas mixture $N_2 : O_2 = 19 : 1$	Gas mixture $CH_2=CH_2 : O_2 = 19 : 1$	
O_2 uptake in mm. ³ of 1 g. tissue alone in the 4th and 5th hours	—	197	—
Extra O_2 in presence of glucose (0.025 %)	643	563	12
	Gas O_2 previously $N_2 : O_2$	Gas O_2 previously $CH_2=CH_2 : O_2$	
O_2 uptake in mm. ³ of 1 g. tissue alone in the 6th and 7th hours	—	115	—
Extra O_2 in presence of glucose (0.025 %)	553	435	21

oxygen uptake was the same as that of the controls (Table I). The experiments were carried on for another 2 hours, after adding substrate once more and filling up the apparatus now with pure oxygen instead of the gas mixtures used before. The results were as expected. We observed a decreased oxygen uptake in the vessels which had previously been filled with acetylene, a normal one in those which had contained ethylene or propylene in the previous 2 hours (Table I). This indicates that the inhibitory effect, if it occurs, is an irreversible one. Acetylene is known to be a cell poison, and these irreversible effects may be ascribed to its toxic action on the cells during the prolonged period during which they are exposed to it.

It was found, in further experiments, that after adding as substrate sodium

Table II.

	Gas mixture $N_2 : O_2 = 4 : 1$ 321	Gas mixture $CH \equiv CH : O_2 = 4 : 1$ 342	Inhibi- tion %
O_2 uptake in mm. ³ of 1 g. tissue alone in the 4th and 5th hours			—
Extra O_2 in presence of 0.05M sodium succinate	1969	1680	15
	Gas O_2 previously $N_2 : O_2$ 205	Gas O_2 previously $CH \equiv CH : O_2$ 140	30
O_2 uptake in mm. ³ of 1 g. tissue alone in the 6th and 7th hours			
Extra O_2 in presence of 0.05M sodium succinate	1855	1610	13
	Gas mixture $N_2 : O_2 = 1 : 1$ 320	Gas mixture $CH \equiv CH : O_2 = 1 : 1$ 280	12
O_2 uptake in mm. ³ of 1 g. tissue alone in the 4th and 5th hours			—
Extra O_2 in presence of 0.05M sodium succinate	1820	1990	—
	Gas O_2 previously $N_2 : O_2$ 144	Gas O_2 previously $CH \equiv CH : O_2$ 123	14
O_2 uptake in mm. ³ of 1 g. tissue alone in the 6th and 7th hours			
Extra O_2 in presence of 0.05M sodium succinate	1740	1840	—
	Gas mixture $N_2 : O_2 = 4 : 1$ 332	Gas mixture $CH_3CH_2 = CH_2 : O_2 = 4 : 1$ 310	—
O_2 uptake in mm. ³ of 1 g. tissue alone in the 4th and 5th hours			—
Extra O_2 in presence of 0.05M sodium succinate	1950	1820	—
	Gas O_2 previously $N_2 : O_2$ 203	Gas O_2 previously $CH_3CH_2 = CH_2 : O_2$ 175	15
O_2 uptake in mm. ³ of 1 g. tissue alone in the 6th and 7th hours			
Extra O_2 in presence of 0.05M sodium succinate	2180	2035	—
	Gas mixture $N_2 : O_2 = 19 : 1$ 420	Gas mixture $CH_3CH_2 = CH_2 : O_2 = 19 : 1$ 335	21
O_2 uptake in mm. ³ of 1 g. tissue alone in the 4th and 5th hours			— 230
Extra O_2 in presence of 0.05M sodium succinate	630	2015	— 230
	Gas O_2 previously $N_2 : O_2$ 300	Gas O_2 previously $CH_3CH_2 = CH_2 : O_2$ 195	35
O_2 uptake in mm. ³ of 1 g. tissue alone in the 6th and 7th hours			
Extra O_2 in presence of 0.05 M sodium succinate	2730	2055	25
	Gas mixture $N_2 : O_2 = 19 : 1$ 420	Gas mixture $CH_2 = CH_2 : O_2 = 19 : 1$ 335	21
O_2 uptake in mm. ³ of 1 g. tissue alone in the 4th and 5th hours			— 220
Extra O_2 in presence of 0.05M sodium succinate	460	1465	— 220
	Gas O_2 previously $N_2 : O_2$ 300	Gas O_2 previously $CH_2 = CH_2 : O_2$ 195	35
O_2 uptake in mm. ³ of 1 g. tissue alone in the 6th and 7th hours			
Extra O_2 in presence of 0.05M sodium succinate	2930	2000	32

succinate, instead of glucose, the oxidation was not affected even by the presence of acetylene. (A distinct inhibition, which we once observed, is certainly to be explained by an experimental error.) In the ethylene-oxygen mixture, with its small content of oxygen, we again observed the same stimulating effect of the narcotic as was mentioned above. The oxygen uptake in a mixture which contained only 5 % oxygen was nearly as high as in a mixture with 20 %, and much higher than that in a mixture of 95 % nitrogen and 5 % oxygen. In the following period, when the vessels were filled with pure oxygen, the condition of affairs was reversed. The oxygen uptake in the vessel which previously contained O_2 and N_2 was much higher than in that which previously contained O_2 + narcotic. These results were confirmed with propylene in low concentrations (Table II).

Each experiment was carried out with 0.5 g. mouse brain. The vessels of the Barcroft apparatus contained 3 cc. of the Ringer solution mentioned above, but without addition of substrate. The filling with the gas mixture of the required composition was performed in the same way as in the previous experiments. The manometers were read every 10 minutes, the temperature being about 37°. After 3 hours, 0.3 cc. of a 0.25 % glucose solution (or 0.5*M* sodium succinate solution) was placed in both cups of two of the manometers, two other apparatus remaining without addition of substrate. The vessels were refilled in the same way as before and with the same gas mixtures. After 2 hours' further shaking the same quantity of glucose (or succinate) was added, the gas mixture was removed, and the manometers were filled with pure oxygen. The evacuation and running in of oxygen were repeated twice to remove the previous mixture as completely as possible. The readings were continued for a further 2 hours. The values given in the table are typical of a large number of results, the volumes of oxygen being corrected for normal temperature and pressure.

SUMMARY.

The suggestion of Quastel, that in incubation experiments the solubility of narcotic gases in lipoids may give rise to an apparent uptake of oxygen, is disproved.

Narcotic gases (ethylene, propylene, acetylene) have no inhibitory action on the oxygen uptake of brain tissue, depleted of its substrate by washing with distilled water, to which glucose, lactate or succinate has been added.

Experiments similar to those of Quastel in which, after depletion of initial substrate by preliminary shaking in Ringer solution with the appropriate gas mixture, the oxygen uptake is measured in the presence of ethylene or propylene, show no inhibitory effect of these gases. The inhibition caused by acetylene, which Quastel observed, was confirmed.

On carrying on the investigations for another two hours, and incubating now in pure oxygen, the inhibition continued in those vessels, which had previously been filled with acetylene, while the presence of ethylene and propylene in the two hours prior to these measurements caused no decrease in the subsequent oxygen uptake in pure oxygen. The inhibition described by Quastel, therefore, is an irreversible toxic effect, due to a too prolonged period of exposure to acetylene.

The experiments were done in collaboration with Frl. M. Lerchenthal, whom I wish to thank for her help.

My thanks are due to Dr E. G. Holmes for his help and criticism and to Dr I. H. Page for his interest in this work. The research was sponsored by the Rockefeller Foundation.

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CCLI. THE ISOLATION OF AMINO-ACIDS IN THE FORM OF THE CORRESPONDING CARBAMIDO-ACIDS AND HYDANTOINS.

I. THE DERIVATIVES OF THE MONO-AMINO-MONOCARBOXYLIC ACIDS.

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(Received October 31st, 1933.)

LIPPICH [1906] called attention to the insolubility of α -carbamido-isobutylic acid, prepared by the action of potassium cyanate or urea on leucine, and suggested the use of this derivative for the isolation and characterisation of the amino-acid. Later [1908] he described the carbamido-acids corresponding with glycine, alanine, α -amino-isobutyric acid, valine, leucine, aspartic and glutamic acids, tyrosine and taurine. He also described the anhydrides of some of these carbamido-acids. In a subsequent paper [1914, 1] he discussed further the reliability of the carbamido-reaction as a qualitative test for leucine. In the same year [1914, 2] he described the application of the reaction to the isolation of small quantities of leucine and other amino-acids added to urine and to blood-serum. Dakin [1910, 2] observed the production of carbamido-acids in urine concentrated by heat. Weiland [1912], Embden and Schmitz [1912] and Kondo [1912] found the reaction useful for the isolation of amino-acids from urine. Howbeit until lately, apparently no amino-acid had been isolated in this way from normal or pathological urine unless it had first been fed in quantity to the animal or introduced directly into the blood-stream or the urine itself. Hoppe-Seyler [1933] has obtained a carbamido-hydantoin derivative of lysine from the urine of a cystinuric patient, using a silver precipitation.

Lippich [1914, 1] and Dakin [1918] called attention to the usefulness of the hydantoins derived from the amino-acids for the isolation and characterisation of the latter. Dakin remarks that the ordinary hydantoins are more useful in this respect than the phenylhydantoins or the thiohydantoins. He described methods [1918; 1920] for the purification of proline and hydroxyproline respectively which involve the isolation of these substances as their hydantoin derivatives.

It does not appear from the literature that anyone has converted protein hydrolysates as a whole into the carbamido-acids or hydantoins with a view to the separation of the derivatives of the various amino-acids. In this preliminary paper experiments are described which show that it is easy to isolate a number of amino-acids from protein hydrolysates as the corresponding carbamido-acids in yields similar to those obtained by other methods. The method is applicable to relatively small samples of protein, 25 g. being a suitable quantity.

Perhaps the greatest advantage consists in the certainty of the characterisation of the amino-acids isolated in this way. In particular the melting-points of the carbamido-acids and hydantoins are more characteristic than those of the amino-acids themselves.

In general the hydantoins are less soluble in water than the amino-acids from which they are derived. One part of leucine dissolves in 44 parts of water at 20° whereas one part of the hydantoin requires 400 parts. There is also a general parallelism between the solubilities of the amino-acids and those of the corresponding hydantoins.

The possibility of evolving a scheme for the separation of the α -carbamido-acids and hydantoins from each other is favoured by the solubility of many of these substances in organic solvents.

Various proteins, including caseinogen, cod-muscle protein, gelatin, edestin and haemoglobin were hydrolysed by boiling with hydrochloric acid. The hydrolysate in each case was converted into a mixture of carbamido-acids by removal of the acid as far as possible by concentrating *in vacuo*, neutralising and boiling the mixture of amino-acids with potassium cyanate or urea. After careful acidification of the cold solution to the turning point of Congo red, a precipitate separated out. On recrystallisation of this material, after decoloration of its aqueous solution with charcoal, it was found to consist of a mixture of the carbamido-acids derived from leucine, *isoleucine* (if present in the protein) and phenylalanine. Means were found whereby these were separated from each other in the form of the hydantoins.

The carbamido-acids remaining in solution were separated into two fractions, *viz.* those soluble in alcohol at p_H 4, and those insoluble. The carbamido-acids derived from the monoamino-monocarboxylic acids are soluble in alcohol at this p_H , whereas the greater part at least of the remaining material is relatively insoluble. In some experiments this separation by means of alcohol was carried out before precipitation of the water-insoluble carbamido-acids.

An alternative method of removing the derivatives of some of the monoamino-monocarboxylic acids depends on the solubility of their hydantoins in ether or chloroform. The hydantoins obtained from glycine, serine, cystine, cysteine and tyrosine are however insoluble in these solvents.

By separating the carbamido-acids by means of alcohol at p_H 4, and converting them into hydantoins in the usual way, the derivatives of leucine, *isoleucine*, valine, phenylalanine, alanine, proline and hydroxyproline can be removed by means of ether or chloroform and that of tyrosine obtained in pure crystalline form from the residue insoluble in ether.

In the case of cod-muscle protein the amount of tyrosine so isolated was greater, in the case of caseinogen less, than the values previously recorded. It was found, however, that the extraction of α -carbamido- β -(*p*-hydroxyphenyl) propionic acid at p_H 4 by means of alcohol was not complete since the insoluble fraction still gave a strong Millon's reaction. This may be explicable by the resemblance of the carbamido-acid to the dibasic derivatives since it possesses a phenolic group. Undoubtedly the technique can be improved to give a more nearly quantitative separation of the tyrosine derivative.

Means were sought for the separation of the various hydantoins derived from the monoamino-monocarboxylic acids. To this end the approximate solubilities of the individual hydantoins in various solvents were determined. It is hoped to publish the data obtained at an early date. Peculiarities of solubility which have been found useful are discussed below.

*iso*Butylhydantoin (from leucine) can be extracted, along with most of its

homologues and other substances already mentioned, by means of ether. From the mixture so obtained the leucine, *isoleucine* and phenylalanine derivatives can be separated together by utilising their insolubility in water. Of these three, that of leucine has the lowest solubility in chloroform and small quantities of the other two as well as the derivatives of valine and proline can be removed from larger amounts of leucine by the use of this solvent. Benzene gives a more complete, if more tedious, separation, however, since the hydantoin from leucine is only soluble to the extent of one part in about 25,000 parts of benzene at 20°, the other substances concerned being many times more soluble. A litre of benzene at 20° removes only 0.5 g. of benzylhydantoin but at higher temperatures the *isobutyl*hydantoin becomes more soluble and the separation less complete. The active and racemic varieties of *isobutyl*hydantoin do not show marked differences in solubilities, melting-points or crystalline forms.

*secButyl*hydantoin (from *isoleucine*) is much more soluble in water, ether, chloroform and benzene than are *isobutyl*- and benzyl-hydantoins. *secButyl*- and benzyl-hydantoins can be separated from *isobutyl*hydantoin most completely by means of benzene as mentioned above. Whereas α -carbamido-*secbutyl*acetic acid is less soluble in water than α -carbamido- β -phenylpropionic acid, *secbutyl*hydantoin is much more soluble than benzylhydantoin. By utilising these two solubility relationships alternately, a fairly good separation of racemic *isoleucine* from phenylalanine is obtainable.

Active *secbutyl*hydantoin crystallises from water with H_2O and this property can be used for its precipitation from benzene. A few drops of water added to a solution of active *secbutyl*- and benzyl-hydantoins in benzene, precipitate the hydrate of the former almost completely. Active *isopropyl*hydantoin separates from benzene in the same form as active *secbutyl*hydantoin. The separation of these two substances from one another (whether active or racemic) depends on the greater insolubility of the *secbutyl*hydantoin in water. Alternatively the mixture of the two may be converted into the carbamido-acids and the less soluble α -carbamido-*secbutyl*acetic acid separated by crystallisation from water. Active *secbutyl*hydantoin can sometimes be made to crystallise from water in large flattened needles which may be 2 cm. in length. These can be picked out with forceps and wiped clean. A specimen can thus be obtained pure without using benzene. It is also possible to crystallise a mixture of the leucine and *isoleucine* derivatives from water in such a way that a crop of the former separates out first and the mother-liquor after filtration deposits needles of the latter free from the isomeride. As pointed out by Dakin and Dudley [1914] progressive racemisation of the hydantoins occurs when they are heated with mineral acids but more slowly than in alkaline solution. If the conversion of the carbamido-acids into the hydantoins be carried out with due care little racemisation occurs, but the possibility that both the active and racemic forms may be present must be considered. Of course racemisation may occur under certain conditions of hydrolysis of the protein and also in the conversion of the amino-acids into carbamido-acids by boiling the hydrolysate with urea under alkaline conditions.

Benzylhydantoin (from phenylalanine) can be separated from *isobutyl*hydantoin by means of benzene as described. Its low solubility in water (1 : 500) provides a convenient means of separating it from the other ether-soluble hydantoins.

p-Hydroxybenzylhydantoin (from tyrosine) is almost insoluble in cold water, ether and chloroform. It is fairly soluble in alcohol and in acetone and to a small extent in ethyl acetate. On acetylation of the phenolic group a substance soluble in ether is obtained.

Methylhydantoin (from alanine) is very soluble in water and in alcohol, moderately soluble in ether. It can be obtained in quantity from silk fibroin by hydrolysis of the protein, conversion of the amino-acids into hydantoin and extraction of the methylhydantoin with ether in a continuous extractor.

Ordinary hydantoin is insoluble in ether and only moderately soluble in alcohol. It can be precipitated in quantity from the mixture of hydantoins obtained as above from silk fibroin by adding alcohol to the concentrated solution. It can then be partially purified from the salts, which are also precipitated by the alcohol, by extraction with hot glacial acetic acid from which the hydantoin crystallises on cooling. A further purification can be effected by extraction with acetone in a Soxhlet apparatus. A separation of glycine and alanine from each other is thus available which should find use as an adjunct to the ester method in which these two amino-acids may be obtained in the same fraction. Hydantoic acid is less soluble in alcohol than hydantoin, and this property has also been found useful.

The hydantoin from proline has a similar solubility in water to that from valine but it is much more soluble in chloroform and in benzene than the latter. Dakin [1918] described the purification of proline by extraction of the carbamido-acids by ether, that derived from proline being insoluble, followed by conversion of the proline derivative into the hydantoin and extraction of the latter by ether. This process succeeded no doubt under the conditions obtaining, but it is necessary to point out that α -carbamido-isobutylacetic acid is only soluble to the extent of one part in 50,000 parts of wet ether [Lippich, 1914, 1] and so could not be removed as such from the proline derivative by that solvent. It is suggested here that in Dakin's experiments no leucine was present, or the acidity of the solution was such that the hydantoin was slowly formed in the case of the leucine derivative, while the proline derivative remained unaltered. Lippich [1914, 1] criticises the statements of Weiland [1912] and Embden and Schmitz [1912] that the carbamido-acids are soluble in ether. He gives the ether-solubility of the phenylalanine derivative as 1:4000 and that of the tyrosine derivative as 1:3000.

The hydantoin from hydroxyproline is more soluble in water than that from proline and much less soluble in benzene and in chloroform than the latter, benzene being particularly useful for the separation of the two. The high solubility of the hydroxyproline derivative renders it unsuitable for final isolation and weighing of the amino-acid in this form. Dakin [1920] extracted the hydantoin from hydroxyproline with ether and reconverted it into the amino-acid.

Hydroxymethylhydantoin (from serine) has a similar solubility in water to the hydantoin from proline, but it is insoluble in ether and in chloroform. On acetylation a substance soluble in ether is formed.

β -Methylmercaptoethylhydantoin (from methionine) is very soluble in water, and also in chloroform. It is fairly soluble in ether and slightly soluble in cold benzene.

Dithiomethyldihydantoin (from cystine) is insoluble in water, alcohol, ether, chloroform and benzene. The tyrosine derivative, being soluble in alcohol, can be separated from it by that solvent.

Thiolmethylhydantoin (from cysteine) has not been obtained crystalline. It appears to be very soluble in water and in alcohol, but only slightly soluble in ether, in chloroform and in benzene.

It will be observed that a means of fractionating the sulphur of proteins is available owing to the differences in the properties of these hydantoins.

The hydantoin of the dicarboxylic amino-acids are insoluble in alcohol at p_H 6-7. If ammonia be added to an alcoholic solution of hydantoinpropionic acid the ammonium salt is precipitated above p_H 6 but redissolves as the p_H value of the solution is raised. A precipitate again forms (other than ammonium chloride) on bringing the p_H of the solution back to the region of neutrality. Since the hydantoin derived from the monoaminomonocarboxylic acids are soluble in alcohol at neutrality a method of separating the two groups is available. Unfortunately the presence of the derivatives of histidine and of arginine introduces complications. These substances have basic groups which do not react with potassium cyanate, and so they are capable of forming salts with the hydantoin derivatives of the dicarboxylic amino-acids. It appears that salts of this kind may be soluble in alcohol.

Hydantoinacetic acid (from aspartic acid) is very insoluble in water but crystallises slowly; the glutamic acid derivative is much more soluble. Both are insoluble in ether, chloroform and benzene. The hydantoin of pyrrolidone-carboxylic acid is slightly soluble in these three solvents at 20°.

δ -Carbamido-*n*-butylhydantoin (from lysine) has been studied lately by Hoppe-Seyler [1933]. It is moderately soluble in water, sparingly soluble in alcohol and insoluble in ether.

The arginine derivative is soluble in water, but insoluble in alcohol and in ether. The hydrochloride has similar properties.

Glyoxaline-4 (or 5)-methylhydantoin (from histidine) is very soluble in water and in alcohol and slightly soluble in wet ether. The hydrochloride behaves similarly. The hydantoin has not been prepared in the pure condition; an acetyl derivative has however been obtained.

Besides these substances the hydantoin derived from *nor*leucine, *nor*valine, α -aminobutyric acid, α -aminoisobutyric acid, tryptophan and other amino-acids have been studied to some extent.

EXPERIMENTAL.

Preparation of carbamido-acids and hydantoins from amino-acids and determination of their solubilities.

Various carbamido-acids and hydantoins were prepared from pure amino-acids for the determination of melting-points and solubility data.

The amino-acid was heated in solution with $1\frac{1}{2}$ equivalents of potassium cyanate for 30 minutes. The solution was then cooled and carefully acidified with dilute hydrochloric acid to the turning point of Congo red. If the carbamido-acid were sufficiently insoluble in water it was filtered off and recrystallised from water.

If the carbamido-acid could not be obtained readily in crystalline form, excess of hydrochloric acid was added and the solution was evaporated to dryness on the water-bath. The residue was extracted with alcohol or in some cases with ether, chloroform or hot benzene. The solution so obtained was evaporated to dryness, and the hydantoin was crystallised twice from water. In some cases the separated carbamido-acid was used for preparation of the hydantoin.

Solubility determinations were carried out as follows. A hot saturated solution of the derivative was cooled and kept at 20° for 24 hours. In cases where a copious precipitate was deposited at once when the hot solution was cooled, this time was shortened to a few hours. The separated material was filtered off and a weighed or measured quantity of the liquid (between 1 and 5 cc.) was evaporated to dryness in a tared dish. The residue was then dried and weighed. Throughout this work nitrogen determinations were carried out by the micro-Kjeldahl method and the melting-points given are uncorrected.

The isolation of the derivatives of leucine, isoleucine, phenylalanine and tyrosine from cod-muscle protein.

Cod-muscle protein was prepared as described for halibut-muscle protein by Osborne and Heyl [1908]. The protein (25 g. calculated on the dry ash-free basis) was hydrolysed by boiling with 250 cc. of 22 % hydrochloric acid for 40 hours. The acid was then removed as far as possible by evaporation *in vacuo*, and the residue was dissolved in 250 cc. of water. The solution was filtered and neutralised to litmus with sodium hydroxide solution. 30 g. of potassium cyanate were added, and the solution was boiled gently for 2 hours and then concentrated to a volume of approximately 100 cc. Sulphuric acid (25 %) was added to the cooled liquid until the excess of cyanate had been destroyed and the liquid was neutral to Congo red. On addition of 400 cc. of 95 % alcohol with stirring, much material dissolved in the alcohol whilst a pasty mass remained undissolved. The alcohol was decanted off and a fresh quantity added with vigorous stirring. The mass of salts and carbamido-acids was in this way extracted with about 1500 cc. of alcohol in five portions which were all decanted off and united. The alcohol deposited a little insoluble syrupy material on standing and was decanted from this next day.

A little water was added to the alcohol and the solution concentrated *in vacuo* to 100 cc. A precipitate separated out and was filtered off next day and recrystallised from water. It yielded 2.9 g. of material melting at 203° and containing 15.83 % of nitrogen. A further quantity of 0.7 g. of material was obtained in two crops melting at 197° and 180° respectively. The substances crystallised in needles quite insoluble in ether.

α -Carbamido-isobutylic acid prepared from commercial *l*-leucine (found: N, 10.71 %, calc. 10.69 %) melted at 201–202° both in an open and closed capillary and contained 16.05 % N (calc. 16.09 %). The derivative of purchased *dl*-leucine (10.75 % N) melted at 195° both in an open and in a closed capillary and contained 16.01 % N. Lippich [1908; 1914, 1] found the melting-point of the active carbamido-acid to be 189–190° in a closed capillary and 200–210° in an open capillary. Dakin [1910, 1] found it to be 205–206°. Pinner and Spilker [1889] found 200° for the melting-point of the *dl*-carbamido-acid.

The isolated material was dissolved in hot alcohol, 20 cc. of 5 % hydrochloric acid were added, and the solution was evaporated to dryness on the water-bath. The residue was extracted repeatedly with small quantities of chloroform, and the insoluble portion was crystallised from water. Thereafter it weighed 1.8 g., melted at 209° and contained 17.92 % N. Under the microscope it appeared as thin plates, and macroscopically it was a light flaky powder like leucine itself.

The hydantoin prepared from pure *l*-leucine was similar in appearance. It melted at 212° in an open capillary and contained 17.98 % N (calc. 17.95 %). A mixture of the two substances melted at 210°. The hydantoin prepared from *dl*-leucine melted at 209°. Dakin [1910, 1] gives 212° and Pinner and Lifschütz [1887] 209–210° for the melting-points of the active and racemic hydantoins respectively. The hydantoin relatively insoluble in chloroform was therefore the derivative of ordinary leucine. The properties of the derivatives of *isoleucine* and *norleucine* are described below.

The chloroform solution was evaporated to dryness and the residue crystallised from water. A crop of needles separated and was filtered off. It weighed 0.25 g. The mother-liquor was concentrated and a further crop of large needles crystallised along with a little *isobutylhydantoin*. Many of these needles, which were over 1 cm. in length, were separated by means of forceps and wiped clean. When dried in the desiccator they weighed 0.6 g. The residue was evaporated to dryness together with the aqueous mother-liquor from the crystallisation of the material insoluble in chloroform. The dry residue was treated with hot benzene and the suspension filtered when cold. The insoluble matter was recrystallised from water. It melted at 206° and the melting-point was unaltered when the substance was mixed with *isobutylhydantoin*. It weighed 0.09 g. On evaporation of the benzene and crystallisation of the residue from water a mixture of needles and hexagonal plates was obtained, which after being dried in the desiccator weighed 0.3 g.

The substance which crystallised in needles agreed in all respects with the hydantoin prepared from active *isoleucine* and described below. It melted at 95° when heated rapidly. If the finely powdered substance were heated slowly it lost water of crystallisation without melting completely and then melted between 120° and 130°. The hydrated compound contained 16.12 % N (calc. 16.09 %).

α -Carbamido-*sec*butylacetic acid has not been described hitherto. 0.3 g. of commercial *d*-*iso*-leucine (10.71 % N) was boiled with excess of potassium cyanate in 5 cc. of water for 15 minutes. When the cooled liquid was acidified carefully with dilute sulphuric acid the carbamido-acid was precipitated. This was filtered off and recrystallised from water. It crystallised in cubes and long flattened prisms and melted at 205°. It contained 15.95 % N (calc. 16.09 %). Its solubility in water at 20° was 1:700 approximately, whereas that of α -carbamido-*isobutyl*acetic acid is given as 1:1700 by Lippich [1908]. A mixture of equal parts of the carbamido-acids from leucine and *isoleucine* melted at 201° in an open capillary.

sec-Butylhydantoin has not been described hitherto, but Lippich [1908] probably obtained it without being aware since he observes that *isobutyl*hydantoin crystallises from ether in needles. The presence of *isoleucine* in the leucine used by him for the preparation of carbamido-*isobutyl*acetic acid accounts for various discrepancies in melting-points and solubilities recorded by that author.

The hydantoin prepared from commercial *d*-*isoleucine* crystallised in long needles which gave up water of crystallisation on fusion. The hydrated product melted at 95° when the capillary containing the substance was suddenly immersed in a bath at that temperature or when a small crystal was placed in the capillary instead of the finely powdered material and the temperature raised in the usual way. When the powdered substance was heated slowly it melted between 120° and 130°. After careful fusion a melting-point of 150° was found with slight softening at 145°. Amongst the needle-like crystals which composed the bulk of the preparation a few hexagonal and rhombic plates were visible under the microscope. When the hydantoin was dissolved in dilute alkali and the solution warmed for some time, the hydantoin recovered by acidifying with excess of hydrochloric acid and boiling crystallised almost entirely in these plates. Dakin [1910, 1] has shown that the active hydantoins are readily racemised in alkaline solution. *iso*-Propyl- and benzyl-hydantoins show a similar change in crystalline form, when the optically active substances are kept in alkaline solution for some hours. Racemisation also occurs gradually when the active hydantoins are heated with concentrated hydrochloric acid.

The needle-like crystals of hydrated active *sec*butylhydantoin contained 15.93 % N (calc. 16.09 %). The substance crystallised from hot dry benzene in plates. If a few drops of water be added to a solution of active *sec*butylhydantoin in cold benzene a flocculent precipitate of the hydrated compound is thrown down in the form of extremely fine filaments. These evidently lose water readily since some of this material dried at 40° contained 17.66 % N. The same material dried at 150° for 3 hours contained 17.50 % N. Probably slight decomposition occurs at this temperature with loss of nitrogen.

In order to remove any suspicion that the *norleucine* derivative might be present in the insoluble carbamido-acid fraction the carbamido-acid and hydantoin derived from that amino-acid were prepared by synthesis from *n*-hexoic acid. The acid was brominated by means of bromine and amorphous phosphorus and the product was poured into water and washed repeatedly with water. The α -bromo-*n*-hexoic acid was heated with large excess of concentrated ammonia in a closed vessel at 100° for 4 hours. In this way 30 g. of *n*-hexoic acid yielded 19.5 g. of *norleucine* containing 10.87 % N (calc. 10.69 %). The phenyl *isocyanate* derivative melted at 137° and the phenylhydantoin at 110°. The phenyl *isocyanate* of *l*-*norleucine* melts at 133° and the phenylhydantoin at 110–111° [Abderhalden and Heyns, 1933].

The carbamido-acid melted at 176° and contained 15.86 % N (calc. 16.09 %). Kondo [1912] found the melting-point of the inactive α -carbamido-*n*-hexoic acid to be 165°. One part of the carbamido-acid dissolved in approximately 375 parts of water at 20°. α -Carbamido-*n*-hexoic acid can therefore be separated from the leucine derivative and less completely from that of *isoleucine* by crystallisation from water. It would not appear in the insoluble carbamido-acid fraction unless present in considerable quantity in the protein.

The hydantoin melted at 139° and contained 17.78 % N (calc. 17.95 %). One part of *n*-butylhydantoin dissolves in approximately 200 parts of water at 20°.

The quantities of α -carbamidobutylacetic acids, *isobutyl*- and *secbutyl*-hydantoins isolated correspond to 10.2 g. of leucine-*isoleucine* or 6.1 g. of leucine and 3.6 g. of *isoleucine* per 100 g. of protein.

The mother-liquors from which the insoluble carbamido-acids from leucine and *isoleucine*

were first separated and that from which they were recrystallised were united, acidified with excess of concentrated hydrochloric acid and concentrated on the water-bath to a syrup. This syrup was stirred repeatedly with fresh quantities of ether until very little material was being removed each time. The ether was distilled off and the residue crystallised from water. In this way a crystalline fraction and a syrup were obtained. The former might be expected to consist chiefly of hydantoins from phenylalanine, leucine, *isoleucine*, valine and proline and the latter of those from valine, proline, hydroxyproline and alanine in so far as the related amino-acids were present in the protein.

The crystalline ether-soluble hydantoins were recrystallised from water. A relatively insoluble product was obtained which melted at 190–191°, contained 15.53 % N, and weighed 0.55 g. The greater part of this material was in short thick prisms which were transparent at first but became opaque as they dried in a desiccator. When the substance was strongly heated in a dry test-tube it emitted the odour of phenylacetaldehyde, and on ignition it burned with a smoky luminous flame. From these observations together with solubility considerations it was evidently a mixture of benzyl- and *isobutyl*-hydantoins. 0.2 g. of the dry material was therefore suspended in 500 cc. of benzene. The benzene suspension was warmed for some time, cooled and kept at room temperature overnight. The insoluble matter was then filtered off, washed with cold benzene, dried and weighed. It weighed 0.05 g. and melted at 197–202°. After recrystallisation from water it melted at 206°. A mixture with pure *isobutyl*hydantoin melted at the same temperature. The benzene filtrate was shaken repeatedly with ammoniacal water and the aqueous solution was concentrated to small volume. As the concentrated solution cooled a substance crystallised out in rectangular platelets. This material melted at 189–190° and contained 14.79 % N (calc. for benzylhydantoin 14.73 %). Wheeler and Hoffman [1911] give the melting-point of *dl*-benzylhydantoin as 190° and Dakin and Dudley [1914] give 195–196°. The active variety melts at 181–183° [Dakin and Dudley, 1914]. Concentration of the ammoniacal solution causes racemisation of the hydantoin.

The amount of benzylhydantoin in the mixture corresponds to a yield of 1.4 g. of phenylalanine from 100 g. of protein, and the additional *isobutyl*hydantoin raises the yield of leucine to 6.6 g. from 100 g. of protein.

The syrupy residue from which the benzylhydantoin and other hydantoins had been extracted by means of ether was diluted to about 100 cc. and kept for some days. It gradually deposited large spherical aggregates of a substance which was very insoluble in water and in ether, gave Millon's reaction strongly and after recrystallisation from hot water melted at 234° and contained 13.80 % N (calc. for $C_{10}H_{10}O_3N_2$, N 13.59 %). *p*-Hydroxybenzylhydantoin melts at 242–245° in a closed capillary [Lippich, 1908]. Wheeler and Hoffman [1911] give the melting-point of *dl*-*p*-hydroxybenzylhydantoin as 257–258°. The amount of material isolated was 0.85 g. corresponding to 3.0 g. of tyrosine from 100 g. of the protein.

The yield of tyrosine obtained by crystallising the amino-acid itself from a hydrolysate of 150 g. of cod-muscle protein was 2.3 g. per 100 g. of protein, a figure which agrees with that of Osborne and Heyl [1908] for halibut-muscle protein. However, Millon's reaction showed the presence of much tyrosine in the mother-liquors.

The melting-point of the isolated material was raised to 247° by recrystallisation from alcohol.

*The isolation of the derivatives of leucine, isoleucine, valine,
phenylalanine and tyrosine from caseinogen.*

As in the case of cod-muscle protein 25 g. of the protein (commercial caseinogen), calculated on the dry ash-free basis, were used. The method differed from that described above in that the water-insoluble carbamido-acids were precipitated and filtered off before extraction of the derivatives of the monoaminomonocarboxylic acids by means of alcohol at p_H 4.

The precipitate of insoluble carbamido-acids was decolorised by means of animal charcoal and recrystallised from hot water. Two different experiments gave almost the same yields of a mixture of α -carbamido-*isobutyl*acetic and α -carbamido-*secbutyl*acetic acids corresponding to 9.3 g. of leucine-*isoleucine* per 100 g. of protein. The mixture melted at 197° and contained 16.04 % N.

0.75 g. of this material was dissolved in dilute alcohol. A few cc. of concentrated hydrochloric acid were added, and the solution was evaporated to dryness on the water-bath to effect con-

version into the hydantoin. The residue was dried at 100° and then treated with 500 cc. of hot benzene. The benzene suspension was cooled and filtered after some hours. The insoluble material was dissolved in hot alcohol, the solution filtered, diluted with water and concentrated. 0.48 g. of hydantoin were obtained by crystallisation. This melted at 208° and contained 18.02 % N. It was therefore pure *isobutylhydantoin*.

The benzene filtrate was shaken repeatedly with fresh quantities of ammonia water and the aqueous extracts were evaporated to dryness. The residue weighed 0.15 g. It was dissolved in a little hot benzene, the solution was filtered and the filter-paper was washed with hot benzene. The precipitate which separated as the benzene cooled was filtered off and dried for 3 days at 90°. It weighed 0.12 g. and melted at 145–150° and contained 17.50 % N. It crystallised from water partly in platelets, partly in needles and was evidently a mixture of active and racemic *secbutylhydantoin*. The slightly low N content is due to the difficulty of expelling the last traces of water from the active material.

A further 0.04 g. of active *secbutylhydantoin* was obtained from the aqueous mother-liquor from which the *isobutylhydantoin* had been recrystallised by precipitation from benzene on addition of a drop of water. This melted at 100° when rapidly heated in the manner of hydrated *secbutylhydantoin*.

The proportion of leucine to *isoleucine* derivative in the insoluble carbamido-acids was 4:1 corresponding to 7.6 g. and 1.7 g. of leucine and *isoleucine* respectively per 100 g. of protein. Approximately this proportion of *isoleucine* has been found in caseinogen by various workers.

The ether-soluble hydantoins, obtained as described for cod-muscle protein, were crystallised and recrystallised from water. A relatively insoluble fraction was obtained which contained 15.47 % N. This was separated into *isobutyl*- and *benzylhydantoin*s by means of benzene as described for cod-muscle protein. The weight of the mixture was 0.85 g. and the proportion *isobutylhydantoin*:*benzylhydantoin* was 1:3, corresponding to 0.7 g. (8.3 g. altogether) of leucine, and 2.2 g. of phenylalanine per 100 g. of protein.

A second fraction of the ether-soluble hydantoins, more soluble in water than the first, was apparently a mixture of the derivatives of *isoleucine* (active and racemic), phenylalanine and leucine, but was not fully investigated.

The remainder of the ether-soluble material was concentrated to a sticky consistency and was rubbed up with 500 cc. of hot benzene. When the benzene was filtered and allowed to stand it deposited a precipitate which consisted of a felt of fine needles. This was filtered off and recrystallised from benzene. After being kept in a desiccator for some days it was found to contain 18.9 % N. The melting-point of the finely divided material was indefinite and appeared to vary from 80° to 130° according to the time of drying and the rate of heating. It crystallised from water in long needles which melted at 112°. One part dissolved in 40 parts of water at 20°.

dl-Valine was synthesised from *isovaleric acid* in the manner already described for *norleucine*. The valine contained 11.75 % N (calc. 11.96 %). The carbamido-acid contained 17.20 % N (calc. 17.50 %). The hydantoin contained 19.77 % N (calc. 19.71 %). The carbamido-acid melted at 187°. Lippich [1908] found the melting-point to be 176° in a closed capillary. The hydantoin melted at 143°. The hydantoin prepared from commercial *dl*-valine melted at 145°. Lippich [1914, 1] gives the melting-point of the hydantoin in a closed capillary as 132°.

The racemic hydantoin crystallised from water in hexagonal and rhombic plates similar to those of *dl-secbutylhydantoin*. One part of the hydantoin dissolved in approximately 23 parts of water at 20°, 130 parts of ether, 130 parts of chloroform and 2000 parts of benzene.

Some *l*-valine was purchased. It contained 12.15 % N. The hydantoin prepared from it was soluble in hot benzene from which it separated in a felt of fine needles. These melted indefinitely up to 143° according to the time of drying and the rate of heating in determining the melting-point. Nitrogen determinations carried out on this substance were lower (18.74 %) than the formula $C_5H_{10}O_2N_2$ requires (19.71 %). After racemisation, however, the nitrogen content of material crystallised from benzene and dried at ordinary temperature was 19.89 %, and the melting-point was 145°. One part of active *isopropylhydantoin* dissolves in approximately 32 parts of water at 20°.

The amount of *isopropylhydantoin* separated from caseinogen as described was 1.2 g. corresponding to a yield of about 4 g. of valine per 100 g. of protein.

That fraction of the hydantoins which was insoluble in ether but derived from carbamido-acids soluble in alcohol at p_{H+4} deposited a precipitate of *p*-hydroxybenzylhydantoin in microscopic needles. When this was filtered off and recrystallised from dilute alcohol it melted at 248° and contained 13.79 % N. It weighed 0.80 g. corresponding to a yield of 2.9 g. of tyrosine per 100 g. of protein.

The isolation of the derivatives of leucine, phenylalanine and proline from gelatin.

Some proteins present an easier problem than others. In the case of gelatin the absence of isoleucine and valine in considerable quantities permits a comparatively easy separation of the hydantoin of proline.

A gelatin hydrolysate was made slightly alkaline with sodium hydroxide solution and boiled with urea equal in weight to the gelatin used. The solution was then made strongly acid by addition of concentrated hydrochloric acid and was boiled for 3 hours. (In the presence of urea and salts the formation of the hydantoins from the carbamido-acids takes place more slowly than in the case of pure carbamido-acids.) The solution of hydantoins was extracted with ether in a continuous extractor for some days. A mixture of hydantoins was obtained from which the derivatives of leucine, phenylalanine and proline were obtained pure by recrystallisation from water and the use of benzene as already described. The proline derivative contained 20.09 % N (calc. 20.0 %), and melted at 157° . When the isolated compound was mixed with a preparation made from pure proline picrate (m.p. 148°) and melting at 165° , the melting-point of the mixture was 161° .

There is no doubt that in the case of gelatin the yield of these three derivatives could be made practically quantitative, but in view of the desirability of proceeding with other parts of the work the experiment was discontinued.

The isolation of the derivatives of tyrosine, glycine and alanine from silk fibroin.

The protein (50 g. of silk waste calculated on the dry ash-free basis) was converted into a mixture of hydantoins in the manner described for gelatin. Potassium cyanate was however used instead of urea.

On concentration of the solution to 300 cc. about 3.5 g. of dark-coloured *p*-hydroxybenzylhydantoin separated and were filtered off. It gave an intense Millon reaction. The filtrate was concentrated to a syrup and treated with 300 cc. of absolute alcohol. A mixture of salts and organic matter was precipitated and was filtered off after several hours. The new filtrate was concentrated *in vacuo* and the residue was again treated with 300 cc. of absolute alcohol. More organic material separated, crystallising on the side of the beaker where it had been scratched. This was also removed by filtration. A third crop was obtained in like manner. The separated material was treated with 350 cc. of hot glacial acetic acid, and the liquid was filtered hot. The insoluble salts were washed repeatedly with hot glacial acetic acid, and the washings were added to the rest of the acetic acid solution. As the solution cooled a crystalline substance separated and was filtered off. A further quantity was obtained on evaporation of the acetic acid. The crude product was boiled in solution with dilute hydrochloric acid in order to reverse any acetylation which might have occurred. The solution was then evaporated to dryness, and the residue was extracted with acetone in a Soxhlet apparatus. After crystallisation from water the extracted material melted at 222° and contained 27.81 % N (calc. for hydantoin 28.0 %). A mixture with pure ordinary hydantoin (m.p. 220°) melted at the same temperature. The weight of hydantoin thus isolated was 14 g. corresponding to 21 g. of glycine per 100 g. of protein.

An unsuccessful attempt was made to separate the rest of the glycine and alanine from the mother-liquor as the carbamido-acids. For this purpose the solution of hydantoins was boiled with dilute alkali. This line of attack was abandoned, however, and the hydantoins were reformed in the racemic condition.

The mother-liquors were concentrated and extracted with ether in a continuous extractor. Small amounts of benzyl- and *sec*butyl-hydantoins were removed in the first few hours; thereafter a hydantoin very soluble in water collected in the receiver. The extraction was slow, occupying a period of many days, and the experiment was discontinued when about 6 g. of substance had been collected. This was crystallised from water, from which it separated, with water of crystallisation, in microscopic dumb-bell or kidney-shaped aggregates. It melted at 143° and contained

24.45 % N after being dried at 100°. Methylhydantoin requires 24.56 % N. Königs and Mylo [1908] found that the racemic hydantoin melted at 146.5°.

The amount of methylhydantoin isolated corresponded to 9 g. of alanine per 100 g. of protein, but considerably more could have been obtained if the extraction had been continued.

DISCUSSION OF RESULTS.

Whether it be considered by itself, or in conjunction with previously existing methods of protein analysis, the carbamido-method shows promise of considerable utility.

It should prevent the possibility of mixtures of commonly occurring amino-acids being mistaken for hitherto unknown substances.

Mixtures of leucine, *isoleucine* and valine, of tyrosine and cystine, and of glycine and alanine have been found difficult to resolve. The methods described or indicated here provide solutions of these problems.

The use of urea instead of potassium cyanate in conjunction with these methods of separation provides a cheap process for the preparation of certain amino-acids in the pure condition. The hydrolysis of the hydantoins, however, yields only racemic amino-acids.

SUMMARY.

1. The properties of the carbamido-acids and hydantoins corresponding to various amino-acids present in protein hydrolysates have been studied with reference to methods of characterisation and isolation of these amino-acids.

2. Methods of isolating leucine, *isoleucine*, valine, phenylalanine, tyrosine, proline, alanine and glycine from protein hydrolysates have been described.

In conclusion I wish to thank Dr W. Robson, Miss E. M. Hill and Miss A. Shore for gifts of various amino-acids. I have much pleasure in acknowledging the encouragement and advice which I have received from Dr Robson in carrying out this work.

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CCLII. THE DEHYDROGENASES OF *BACTERIUM COLI*.

I. THE EFFECT OF DILUTION: WITH A NOTE ON THE EXISTENCE OF A CO-ENZYME OF GLUCOSE DEHYDROGENASE.

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(Received October 31st, 1933.)

THE activity of an enzyme preparation is normally proportional to the amount of enzyme present. Quastel and Whetham [1924] showed that this proportionality existed in the case of the succinic dehydrogenase of *Bact. coli*. Although this was the only dehydrogenase studied, and although the dilution was made only over a small range, it has since been assumed that such proportionality holds for all the dehydrogenases, and hence, that over a wide range of reduction times the reciprocal of these times is a true measure of the amount of the enzyme under investigation.

During a study of the effect of poisons on the dehydrogenase systems of *Bact. coli*, it soon became evident that such assumptions were not entirely justifiable. The effect of dilution has, therefore, been investigated for three dehydrogenases of this bacterium. The results, recorded below, led to a detailed investigation of each of the several factors involved in the methylene blue technique of Thunberg, as developed by Quastel for the study of the bacterial dehydrogenases. As usually followed, this consists in observing the time of reduction of a given quantity of methylene blue *in vacuo* in the presence of a suspension of washed bacteria by a given substrate at a definite temperature in the presence of a buffer solution.

EXPERIMENTAL.

A washed suspension of *Bact. coli* was used. The bacteria were grown in Roux bottles for 15–18 hours on a tryptic digest of caseinogen. They were centrifuged, washed twice by centrifuging in distilled water and finally made up in uniform suspension in distilled water. Such a preparation showed no appreciable differences in the properties of the dehydrogenases here investigated from one in which the bacteria were suspended in Ringer solution instead of distilled water. The enzymes studied were formic acid, succinic acid and glucose dehydrogenases. The normal procedure, which in several experiments has been modified as described later, was as follows. 1 cc. each of distilled water, phosphate buffer p_H 7.0 ($M/20$), methylene blue (1/5000), substrate solution and bacterial suspension were placed in a Thunberg tube. The final volume was always 5 cc. The tube was evacuated for about $1\frac{1}{2}$ minutes at the water-pump, then placed in a bath at 40°. This was taken as the beginning of the experiment

and the time taken for the methylene blue to become reduced by 80 % as estimated by comparison with standards was taken as the reduction time. Each experiment was done at least in duplicate. The substrates were *M*/10 solutions of sodium formate, sodium succinate and glucose. The bacterial suspension used was of different concentrations for the different enzymes, being strongest for the succinic and most dilute for the formic dehydrogenase. Thus similar reduction times were obtained for the three dehydrogenases.

Effect of dilution.

If a strict proportionality exists between enzymic activity and dilution, then the product of the dilution and the activity should be constant for all degrees of dilution. The activity of the dehydrogenases may be taken as the reciprocal of the reduction time. If now the product of activity and dilution is plotted against the reduction time, the limits of reduction time between which proportionality holds can be read off directly. In Fig. 1 the results for the three

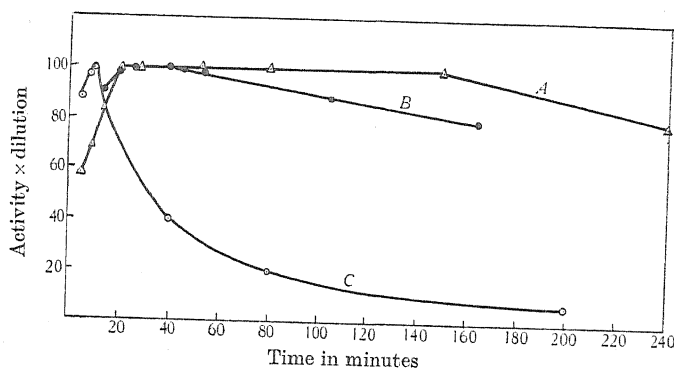


Fig. 1. Effect of dilution on the dehydrogenases.

A. Formic dehydrogenase, Δ . B. Succinic dehydrogenase, \bullet . C. Glucose dehydrogenase, \circ .

enzymes are so plotted. In order that the three curves might be plotted on the same figure, the maximum value of the product of activity and dilution has been given an arbitrary value of 100 units.

Similar curves are of course obtained on plotting the product of activity and dilution against dilution.

It will be seen that formic dehydrogenase exhibits strict proportionality between dilution and activity when the reduction time lies between 20 and 160 minutes. After this, the activity begins to decrease rather more rapidly than is warranted by the dilution. Succinic dehydrogenase shows this proportionality between reduction times of 20 and 50 minutes. With both enzymes, when the reduction time is less than 20 minutes the activity is too low, *i.e.* the reduction time is too long, a result mainly due to the errors introduced by the relatively large temperature coefficient of the reaction effecting an increasing rate until the materials attain the temperature of the bath¹.

Another possible source of error is the reduction of some of the methylene blue before the tube is placed in the bath. The results show clearly, however, that this effect, which would tend to give too low a reduction time, is completely masked by the temperature lag effect.

¹ The presence of small quantities of residual air would have a similar effect.

The results with glucose dehydrogenase are very striking. There is no range at all within which there is any proportionality between activity and dilution. When the reduction time is less than 10 minutes, a rise in the curve occurs as with the other two enzymes. In this case, however, the rise does not continue until a reduction time of 20 minutes is reached and then remain constant, but a rapid fall in the curve occurs when the reduction time exceeds 10 minutes. The effect of factors responsible for the extreme lack of proportionality with glucose dehydrogenase would seem to exceed the temperature lag effect when the reduction time is more than 10 minutes.

The lack of proportionality at the higher reduction times might be due to a gradual destruction of the enzymes at 40° or to a slow poisoning of the enzymes by methylene blue. The phosphate buffer and the substrate itself might also be influencing the reduction times. All these factors have, therefore, been investigated for each of these three enzymes.

Inactivation of the enzymes at 40°. It is well known that on keeping a suspension of *Bact. coli* for some time, even at 0°, there is a decrease in the activity of the enzymes. By far the most susceptible of the three here studied is the glucose dehydrogenase. Quastel and Wooldridge [1927] showed that on keeping a suspension of *Bact. coli* for one hour at higher temperatures (47° and 57°) formic dehydrogenase was unaffected, succinic affected appreciably at 57°, whilst the glucose dehydrogenase was appreciably affected at 47° and completely inactivated at 57°.

It therefore seemed likely that glucose dehydrogenase was being appreciably inactivated at the temperature of the bath (40°) and that this might in part account for the decreased activity noted when the concentration of the bacteria was such as to give a fairly long reduction time.

Suspensions of the bacteria were kept at 40° for one hour and the activities compared with suspensions kept at 0° (Table I). It will be seen that this treatment causes no diminution in the activity of any of the enzymes.

Table I.

Dehydrogenase	Reduction time in minutes after one hour at	
	0°	40°
Formic	17.75	18.25
Succinic	22.5	20.0
Glucose	10.75	10.25

Poisoning by methylene blue. 1 cc. of methylene blue solution was placed in each of a number of Thunberg tubes and 1 cc. of bacterial suspension added. At varying intervals, 1 cc. each of buffer solution, substrate solution and water were added to these tubes, which were then evacuated and the reduction times taken.

The results are given in Fig. 2 in which the original activity of the enzyme has been given a value of 100 units. Formic dehydrogenase is entirely unaffected by methylene blue in 5 hours. Succinic dehydrogenase is poisoned to the extent of 40 % after 2 or 3 hours. Glucose dehydrogenase is poisoned to the extent of 80 %; at first the poisoning is rapid, then it becomes much slower.

The poisoning action of methylene blue in these experiments took place at 19–20°. An experiment with glucose dehydrogenase at 40° showed that the poisoning was much more rapid but the final activity of the poisoned enzyme was the same. Glucose dehydrogenase is also poisoned to the same extent *in vacuo*.

In these experiments, the poisoning action of methylene blue continues during the period of reduction. The times given on the abscissa in Fig. 2 are the times from the addition of the methylene blue to the bacteria to the time when put in the bath with the other substances. These times are, therefore, less than the actual time of action of the methylene blue.

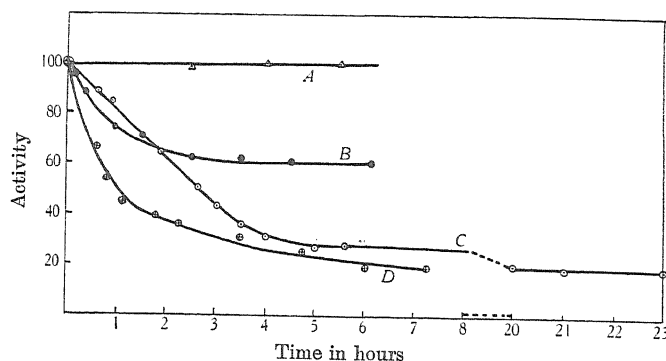


Fig. 2. Poisoning of the dehydrogenases by methylene blue.

- A. Formic dehydrogenase at 19°, Δ . B. Succinic dehydrogenase at 19°, \bullet .
 C. Glucose dehydrogenase at 19°, \circ . D. Glucose dehydrogenase at 40°, \oplus .

Since dilution involves more prolonged action of the dye on the enzyme, the poisoning effect of methylene blue seems at first glance to account for the observed discrepancies in the dilution experiments, since the enzyme most affected by dilution is the one most affected by methylene blue. Formic dehydrogenase, which is little influenced even by great dilution, is unaffected also by methylene blue. The poisoning experiments might also account completely for the falling off in the activity of succinic dehydrogenase when the reduction time is greater than 50 minutes, since even at 19° an appreciable amount of poisoning has taken place in this time. In the case of glucose dehydrogenase, however, even though the enzyme has been poisoned to the extent of 50 % in one hour at 40°, this would hardly account for such a great fall in the activity as that observed when the reduction time is increased from 10 minutes to, say, 25 minutes. It is probable, therefore, that there are other factors which cause this phenomenon in the case of glucose dehydrogenase.

Effect of phosphate buffer. The effect of leaving the bacteria in contact with the phosphate buffer was investigated in a manner exactly analogous to that described for methylene blue. The suspension of bacteria was left in contact

Table II.

Dehydrogenase	Time of action of phosphate on bacteria minutes	Reduction time minutes
Formic	0	18.5
	15	18.5
	35	19.5
Succinic	0	36.0
	120	24.0
Glucose	0	15.5
	40	13.5
	60	11.5
	120	10.5

with the phosphate at room temperature, the other substances added after varying intervals and the reduction time determined (Table II).

Phosphate has very little effect on formic dehydrogenase; if anything, it results in a slight inhibition of the enzyme. The actions of both the succinic and glucose dehydrogenases are accelerated by leaving the bacteria in the presence of the phosphate for a time. Maximum acceleration, about 30 %, is obtained after about 2 hours at a temperature of about 20°.

The activation of succinic and glucose dehydrogenases by phosphate is readily reversed by removal of the phosphate by centrifuging. The activities of the enzymes determined in the ordinary way are found to be the same as before treatment with phosphate (Table III).

Table III.

Dehydrogenase	Reduction time in minutes		
	Initially	After action of phosphate for 2 hours	After removal of phosphate
Succinic	31.0	21	33
Glucose	11.25	8	12

Effect of phosphate buffer on the poisoning by methylene blue. The bacterial suspension was left in contact with both phosphate buffer and methylene blue for an hour and the activities of the enzymes determined after the addition of the substrates.

Table IV.

Dehydrogenase	Reduction time in minutes		
	Initially	After action of methylene blue for one hour	After action of methylene blue and phosphate for one hour
Formic	13.0	13.25	13.25
Succinic	17.5	21.25	21.0
Glucose	8.0	15.5	15.25

It will be evident from Table IV that the presence of phosphate has no effect on the poisoning by methylene blue of any of the enzymes. That is, the accelerating effect of phosphate is not observed in the presence of methylene blue.

Effect of using buffers other than phosphate. Although the effect of phosphates is masked by the presence of methylene blue, it was thought that an acceleration might occur in the ordinary procedure adopted in the methylene blue technique. In an attempt to avoid this possible effect of phosphate, two other buffers were tried. Sodium maleate buffer was prepared according to the description of Temple [1929]. Although Temple describes it as a buffer only for the range p_H 5.2-6.8, it can readily be shown that even at p_H 7.0, it still has about 10 % of its maximum buffering power. It was accordingly prepared and used at p_H 7.0. The second buffer prepared was the diethyl barbiturate (veronal) buffer of Michaelis [1930].

These buffers were used in a concentration of $M/20$ and compared with phosphate buffer. The activities of the enzymes were determined in the ordinary way. In certain experiments, 1 cc. of phosphate solution was added as well as the 1 cc. of the other buffer. The results are recorded in Table V.

Table V.

Dehydrogenase	cc. of buffer used			Reduction time in minutes
	Phosphate	Veronal	Maleate	
Formic	1	0	0	18.25
	1	1	0	18.0
	0	1	0	26.0
	1	0	1	23.0
	0	0	1	25.0
Succinic	1	0	0	22.5
	1	1	0	23.0
	0	1	0	31.0
	1	0	1	27.0
	0	0	1	27.25
Glucose	1	0	0	17.0
	1	1	0	30.0
	0	1	0	28.5
	1	0	1	19.0
	0	0	1	24.0

All three dehydrogenases are inhibited by veronal to the extent of 30–50 %. Glucose dehydrogenase is inhibited by veronal to about the same extent in the presence or absence of phosphate; the other two enzymes, however, have the same activity in the presence of veronal and phosphate as in phosphate alone. Maleate buffer also inhibits all three enzymes, though not equally. Glucose dehydrogenase is much less inhibited if phosphate is present at the same time; formic dehydrogenase is only slightly less inhibited, whilst succinic dehydrogenase shows the same inhibition in the presence or absence of phosphate.

Thus the three enzymes differ considerably in their behaviour towards the three buffers. The exact interpretation of these results is at present impossible, although it seems likely that in some cases phosphate plays an active part in the dehydrogenation.

Effect of acting on substrate. The suspension of bacteria was left for a time in contact with one or other of the substrates. After a time, the other substances were added and the reduction time determined.

(a) *Formic dehydrogenase.* The results with this enzyme were rather variable but in no case was there any significant alteration in the reduction time.

(b) *Succinic acid.* Bacteria left in contact with succinate show an accelerated reduction rate. It is known that succinic acid gives rise by the action of *Bact. coli* [Woelf, 1929] to malic acid, which can act as a hydrogen donator [Quastel and Whetham, 1925]. This would explain the more rapid reduction of the methylene blue after the bacteria have acted for a time on the succinate.

The acceleration due to succinate is slightly less than that due to phosphate. Phosphate and succinate together do not have an additive effect (Table VI).

Table VI.

	Minutes
Reduction time, initial	44
Reduction time, after 30 mins. at 20° with succinate	28
" " succinate and phosphate	30
" " phosphate	31

(c) *Glucose dehydrogenase.* With glucose, as with succinate, a markedly increased activity occurs after the bacteria have been in contact with the substrate for a short time before the addition of the methylene blue. In one experiment, the reduction time determined in the normal way was 13 minutes.

The reduction time after the bacteria had acted on the glucose for 15 minutes at 20° was 9 minutes. An even greater acceleration occurs if the bacteria are allowed to act on the glucose *in vacuo*.

As with succinate, these results may be explained by the formation of a substance or substances which are able to act as additional donators of hydrogen. It is well known that the action of *Bact. coli* on glucose results in the formation of several substances, such as lactic, acetic, succinic and formic acids, ethyl alcohol, *etc.* [Harden, 1901; Grey and Young, 1921; Aubel, 1926]. Of these, formic, succinic, lactic and acetic acids can act as hydrogen donators to methylene blue in the presence of washed suspensions of *Bact. coli* [Quastel and Whetham, 1925]. Moreover, Cook and Haldane [1931] obtained a gradually increasing rate of oxygen uptake for *Bact. coli* in the presence of glucose, which they attributed to the formation of secondary metabolites such as those considered above.

The following experiments are in accordance with the view that the acceleration is due to the production of substances from glucose which can be activated by enzymes of the bacteria to donate hydrogen to methylene blue.

Exp. 1. A suspension of bacteria was left in contact with glucose at three different temperatures. It will be seen from Table VII that no acceleration occurred when the bacteria and glucose were left at 0°, slight acceleration when they were at 20°, and a greater acceleration when they were at 40°.

Table VII.

	Minutes
Reduction time, initial	10.0
Reduction time, after 15 mins. with glucose at 0°	10.25
" " 20°	8.75
" " 40°	6.75

Exp. 2. The bacteria and glucose were left for 30 minutes at 20°, the mixture was then placed in boiling water for one minute; methylene blue was added and the tube evacuated. No reduction occurred after 6 hours.

Exp. 3. A suspension of bacteria was allowed to act on glucose for 30 minutes at 20°. 1 cc. of N/1000 solution of silver sulphate was added¹. Again, no reduction was observed even after 6 hours.

Exp. 4. It has been shown [Quastel and Wooldridge, 1927] that glucose dehydrogenase is completely inactivated by shaking the bacteria for 2 or 3 minutes with toluene. Now the bacteria were allowed to act on glucose for 30 minutes at 20° and were then treated with toluene; it was found that a slow reduction of methylene blue occurs in these conditions. A control experiment, in which the bacteria were treated with toluene before the addition of the glucose, showed no reduction of methylene blue whatever.

From these experiments it is obvious that the action of *Bact. coli* on glucose results in the formation of substances which are capable of being activated as hydrogen donors by enzymes present in the bacteria. Exp. 4 shows that these enzymes are not identical with glucose dehydrogenase.

The effect of glucose on the reduction of methylene blue by *Bact. coli* was similar to its effect with spores of *B. subtilis* [Tarr, 1933]. The reduction time was less after the spores had been left with glucose for 30 minutes at 40°. This was attributed by Tarr to the gradual formation of glucose dehydrogenase in the spores. It seems much more likely that here too there is an accumulation of secondary hydrogen donators, as suggested above for *Bact. coli*. This is sup-

¹ This concentration of silver sulphate has been shown to inhibit completely many dehydrogenases, such as those of glucose, lactic acid and succinic acid.

ported by the observations of the same author that spores treated with ethylurethane cannot reduce methylene blue in the presence of glucose, unless they have been allowed to act on the glucose before the addition of the ethylurethane. This is exactly similar to the results of the treatment of *Bact. coli* with toluene described above. Moreover, *B. subtilis* spores show a gradually increasing rate of oxygen uptake with glucose [Tarr, 1933], which, like the results of Cook and Haldane [1931] for *Bact. coli*, can be best explained by the formation of breakdown products of glucose.

Co-enzyme of glucose dehydrogenase.

It has been pointed out that the poisoning of glucose dehydrogenase by methylene blue is not sufficient to account for the very rapid falling off in the activity of this enzyme on dilution. Moreover, the accelerating effects of the products of the action of the organism on glucose would be expected to result in an increased rate of reduction of methylene blue when the reduction times are longer, and hence these effects would tend to compensate for the poisoning effect of the dye. It is, therefore, evident that the effect of dilution on glucose dehydrogenase is due to some other factor or factors which have not yet been considered.

It was thought possible that there existed in *Bact. coli* a co-enzyme for glucose dehydrogenase, which might be washed out of the bacteria on dilution and so become the limiting factor in the action of the enzyme. This hypothesis was tested by adding, together with untreated bacteria, 1 cc. of a suspension which had been heated to 100° for 5 minutes to the usual reactants in a Thunberg tube. The reduction time of this was taken and compared with the reduction time of glucose dehydrogenase without the addition of heated bacteria. The results are given in Table VIII. There is a considerable acceleration by the addition of heated bacteria, which themselves have no activity.

Table VIII. *Effect of addition of heated bacteria on glucose dehydrogenase.*

Bacteria	Reduction time
Unheated bacteria	33 minutes
Unheated + heated bacteria	11.75 minutes
Heated bacteria	> 6 hours

It was considered possible that the heating of the suspension might have resulted in the liberation of substances which could act as hydrogen donors and hence give an accelerated reduction of methylene blue. That this is not so is shown by the fact that a mixture of heated and unheated bacteria in the absence of glucose causes no reduction of methylene blue even after 3 hours.

The effects of dilution on glucose dehydrogenase in the presence and absence

Table IX.

Concentration of suspension	Without heated bacteria		With heated bacteria	
	Reduction time minutes	Activity \times dilution	Reduction time minutes	Activity \times dilution
1	8	100	8	100
2/3	11	105	12	102
1/2	17	98	17	100
1/3	83	31	22.5	112
1/5	> 360	0	35	117
1/8	> 360	0	72	92
1/12	—	—	125	80
1/16	—	—	240	56
1/24	—	—	500	40
			(approx.)	

of heated bacteria is shown in Table IX. The same undiluted heated suspension was added in each experiment.

The effect of the addition of heated bacteria is very marked in some dilutions. When the bacteria were diluted to 1/5 of their original concentration, the reduction time was too long to be measured; when the heated suspension was present, however, the reduction time was 35 minutes. It will be seen that when the concentration of bacteria is sufficient to give a reduction time of 17 minutes or less, the addition of the heated suspension is without effect. In other words, in these concentrations, there is sufficient co-enzyme present to give the maximum activity.

When the reduction time is greater than 35 minutes there is a rather rapid falling off in activity even in the presence of heated bacteria. This effect might well be due to the gradual poisoning of the enzyme by methylene blue, which was noticed above.

It is evident, therefore, that there is present in *Bact. coli* a thermostable substance capable of acting as co-enzyme to glucose dehydrogenase. The properties of this co-enzyme are now being investigated.

DISCUSSION.

The methylene blue technique as used for the study of bacterial dehydrogenases has been shown to be limited in its applicability. Unless the reduction time is taken between certain limits, which vary for the various dehydrogenases, the activity measured is not a true representation of the strength of the enzyme. For example, with succinic and formic dehydrogenases, when the reduction time is less than 20 minutes, there is an error due to the time taken for the temperature of the reactants to reach that of the bath. The activity is thus too low. This error necessitates a correction to many of the results quoted in the literature.

The upper limit of reduction time for proportionality to hold varies considerably for the different enzymes. Formic dehydrogenase shows a constant "activity \times dilution" up to very long reduction times. Only when the reduction exceeds 2 hours is there a falling off in the activity, and this is very slight. These results are in accordance with the stability of this enzyme to methylene blue and temperature, and the absence of effect of the phosphate buffer.

The results with succinic dehydrogenase, which shows a decreasing activity after 50 minutes, have been explained by the poisoning effect of methylene blue. The accelerating effect due to the action of the organisms on the succinate itself is not sufficient to compensate for this poisoning by the dye.

Glucose dehydrogenase, although poisoned to a greater extent than succinic dehydrogenase by methylene blue, shows a very striking lack of proportionality between activity and dilution. It was found that there is present a thermostable co-enzyme in the bacteria which is probably washed out on dilution. On the addition of a heated suspension of bacteria, the effect of dilution is what one would expect from the poisoning of the enzyme by methylene blue. Here, as with succinic dehydrogenase, it would seem that the poisoning by the dye outweighs the acceleration due to the decomposition of glucose.

SUMMARY.

1. The effect of dilution on activity has been studied for three enzymes of *Bact. coli*, the formic, succinic and glucose dehydrogenases. The product of the activity and dilution, which should be constant, is found to be so within certain limits for the first two of these but not at all for the glucose enzyme.

2. The various factors involved in the methylene blue technique have each been considered in an attempt to explain these discrepancies.
3. None of the enzymes is affected by being kept at 40° for one hour.
4. Formic dehydrogenase is unaffected by being kept for 5 hours with methylene blue or one hour with phosphate buffer.
5. Succinic dehydrogenase is slowly poisoned to a maximum of 40 % by methylene blue. Its action is accelerated when it is kept in presence of phosphate buffer or with succinate.
6. Glucose dehydrogenase is poisoned to 80 % by methylene blue. Its action is accelerated when it is kept with phosphate buffer or with glucose.
7. It is shown by treatment with toluene, silver *etc.*, that the accelerating effect on glucose dehydrogenase produced when the bacteria are kept in contact with glucose is due to the production of substances from the glucose which are activated by the bacteria as hydrogen donators.
8. A thermostable co-enzyme of glucose dehydrogenase has been demonstrated in *Bact. coli*.
9. The effects of dilution on the three enzymes can be explained by the properties of the enzymes studied in this paper.
10. The limitations of the methylene blue technique are discussed.

The author wishes to acknowledge the encouragement and advice received from Prof. E. K. Rideal during the course of this work.

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CCLIII. THE ACCESSORY FACTOR NECESSARY FOR THE GROWTH OF *NEMATOSPORA GOSSYPHII*.

III. THE PREPARATION OF CONCENTRATES OF THE SECOND ACCESSORY FACTOR.

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(Received November 1st, 1933.)

IN an earlier communication [Buston and Pramanik, 1931, 1] it was shown that the "accessory factor" found by Farries and Bell [1930] to be necessary for the growth of *Nematospora gossypii*, the fungus responsible for the "internal boll disease" of cotton, in synthetic media consisted of at least two distinct constituents, one of which was found to be *D*-inositol. The discovery of the necessity for the presence of this compound found a parallel in the observation of Eastcott [1928] concerning "bios" [see also Buston and Pramanik, 1931, 2]. The chemical nature of the second constituent forming the accessory factor was not determined by Buston and Pramanik, who recorded only that it appeared to be precipitable by Neuberg and Kerb's mercuric acetate reagent and might therefore "be associated with the mono-amino-acids."

Since the publication of this earlier work, further attempts have been made to characterise the second factor. Much more active concentrates thereof have been prepared, and although there is as yet no indication that the substance concerned has been obtained in a state approaching purity, it is thought that the results so far obtained may be of interest.

One difficulty has been met with repeatedly; with the very active concentrates, almost any treatment seemed to result in partial destruction of activity, or at any rate in the introduction of some substance giving rise to a "staling" type of growth in the cultures. Even the most extreme care in the use of purified reagents *etc.* has not always succeeded in overcoming this difficulty. Despite this, it has been possible, from a consideration of the reactions given by the active concentrates, to draw some tentative conclusions as to the possible chemical nature of the second factor, although these must be accepted with reserve in view of the admitted impurity of the preparations.

EXPERIMENTAL.

General technique.

The experimental method adopted was that described by Buston and Pramanik; the same strain of the organism was used, and the methods of culture were identical. The standard basal medium was of the following composition: glucose, 2 %; K_2HPO_4 , 0.5 %; $MgSO_4 \cdot 7H_2O$, 0.25 %; nitrogen, supplied from

a standard gelatin hydrolysate, 0.3 %; agar, 2 %. To this was added, where necessary, *i*-inositol (B.D.H. or Kahlbaum), 0.02 %; and the appropriate amount of concentrate of the second factor.

Using their "critical dose" technique, Buston and Pramanik showed that such a dose of concentrate (*i.e.* the amount of concentrate beyond which further addition did not result in a proportionate further increase of growth) gave a growth of 270–290 mg. of mycelium (dry weight from two plates). In general, therefore, in fixing the amount of any concentrate of the second factor required, the "unit dose" was taken as the amount of concentrate, immediately after removal of inositol, necessary to give a growth of 270 mg., the corresponding amounts of the more active concentrates being calculated from the dry-weight yields of the various fractions, due allowance being made for losses at different stages.

Source of the second accessory factor.

The original experiments of Farries and Bell showed that the accessory factor (not then recognised as a mixture) was present in a number of "crude" proteins, and absent from others. The discovery of the dual nature of the accessory factor made it necessary to examine again the latter group of proteins, since it was evidently possible that lack of activity in these was due to lack of inositol only. Further, it was thought that, should any protein be found rich in the second factor, it might serve better as a starting material for its isolation than an indefinite and complex mixture such as the lentil extract of Buston and Pramanik.

With this in view, media were prepared in which the nitrogen (0.3 % N in the medium) was supplied solely in the form of hydrolysates of different proteins; adequate amounts of inositol were added to the media, since this substance was only present as an impurity in some of the crude proteins. The results of these tests are shown in Table I. In the case of every protein except

Table I.

Protein	Growth of mycelium (mg. dry weight)
Gliadin	76
Legumin	104
Edestin	20
Excelsin	65
Zein	124
Oat glutelin	145
Caseinogen	20
Ovalbumin	20
Fibrin	45
Gelatin	0

gelatin there was evidence of the presence of the second accessory factor, although often in very small concentrations only; even fibrin and edestin, stated by Farries and Bell to be completely inactive, contained small amounts of the substance. On the whole, the proteins prepared from seeds apparently contained the greatest amounts of the accessory factor, zein being one of the richest as well as the most easily obtainable. Further samples of zein were prepared, by slightly different methods, and their activities tested; it was found that there was no connection between the activity of a sample of zein and its purity, as measured by nitrogen content (Table II). Further, experiments showed that the most active sample of zein obtained showed, weight for weight,

only two-fifths of the activity of the crude lentil concentrate, so that in spite of objections it seemed best to continue the use of lentils as the source of the accessory factor.

Table II.

Zein, precipitated from dilute alcohol by	Nitrogen in zein	Growth of mycelium
	%	mg. dry weight
Water	16.1	93
Acetone	13.8	365

Fractionation of the lentil concentrate by chemical methods.

(a) *Removal of inositol.* So little evidence was available as to the chemical nature of the second accessory factor that it was hardly possible to devise a logical method of fractionation based upon such evidence. It became necessary therefore to work on somewhat empirical lines, using methods which have found wide application in allied fields of research.

In the first place, some 70 g. of lentil concentrate were prepared, following the method of Buston and Pramanik; from this, the inositol was removed by precipitation with barium hydroxide and alcohol at 0°. Whereas Buston and Pramanik found that one such precipitation sufficed to separate the inositol completely from the second factor, in this case it was found that no sharp separation was obtained at the first precipitation, both the precipitate (inositol) and filtrate fractions needing to be re-fractionated. Even so, a certain amount of the second factor found its way into the inositol fraction, which thus retained some of its activity when tested alone. On the other hand, the filtrate at this stage was free from inositol, and was quite inactive alone, its activity being restored on the addition of pure inositol. A possible reason for the failure to separate the two factors sharply is put forward below (p. 1866). The distribution of activity between the fractions is shown by the results of the plating tests, given in Table III.

Table III.

	Growth of mycelium, per unit dose (mg. dry weight)		
	Precipitated fraction	Filtrate fraction	Filtrate plus inositol
First fractionation	312	245	332
Filtrate refractionated	185	0	224

From 50 g. of crude lentil concentrate, 6.3 g. of solid remained in the filtrate fraction after the second precipitation and was inactive in the absence of inositol. A series of platings using a range of doses of this concentrate showed that the standard growth of 270 mg. was produced in the presence of 25 mg. dry matter per 100 cc. medium (together with 20 mg. inositol).

(b) *Precipitation with mercuric acetate and with phosphotungstic acid.* Buston and Pramanik had reported that the second accessory factor was completely precipitable by the mercuric acetate-sodium carbonate reagent of Neuberg and Kerb. Accordingly the inositol-free fraction of the concentrate was treated with mercuric acetate in the presence of barium hydroxide, this latter substance being used in preference to sodium carbonate in view of the objections to the presence of large amounts of sodium in the concentrates. A 1 % solution of the concentrate was used, and 30 % of the dry matter passed into the mercuric acetate precipitate fraction; the whole of the activity appeared in this fraction.

Platings with a portion of this fraction (after removal of mercury *etc.*) corresponding to 20 mg. of the inositol-free filtrate (*i.e.* 7 mg. of the ppt. fraction at this stage) per 100 cc. medium gave a growth of 269 mg. dry mycelium.

The possibility has been pointed out above, that the second factor might be of the nature of an amino-acid; accordingly, determinations of nitrogen were made on the various fractions of the concentrate. The mercuric acetate precipitate fraction was found to contain 18.1 % N on dry matter, a figure so high as to suggest that basic substances might be present. Accordingly the precipitation of the substance with phosphotungstic acid was studied. A series of precipitations was performed, the concentration of the mercuric acetate precipitate fraction and of the phosphotungstic acid being varied. In all cases precipitation was carried out in 5 % sulphuric acid solution, and a sufficient excess of the reagent was used. The results of growth tests carried out in the usual manner on the several fractions are given in Table IV. From these results,

Table IV.

Solution of con- centrate %	Phosphotungstic acid reagent								
	25 % solution					10 % solution			
	Dry matter		Growth			Dry matter		Growth	
	ppt.	filtr.	ppt.	filtr.	p. + f.	ppt.	filtr.	ppt.	filtr. p. + f.
10	2010	600	251	Trace	286	1355	940	218	Trace 277
4	375	350	168	177	228	425	500	169	160 270
1	377	325	163	157	300	625	525	155	152 286

(Growth figures—mg. dry mycelium.)

it appeared that no sharp separation could be obtained by the use of phosphotungstic acid, although, as shown by the growths given by the recombined fractions, there was little loss of activity. Maximum precipitation, both of active substance and of total dry matter, took place in the stronger solutions both of concentrate and reagent; in the weaker solutions, however, precipitation of total substance and of active material were not always parallel. It seemed therefore that true precipitation, and not a mere adsorption, was concerned. It was concluded that the best method of treating the concentrates, after removal of inositol, would be to precipitate first with the Neuberg-Kerb reagent and then from a solution containing 1 % dry matter with 10 % phosphotungstic acid reagent. By this means, the greater part of the active substance passed into the phosphotungstic acid precipitate, although some 25 % escaped precipitation.

From an original lentil concentrate giving a growth of 275 mg. dry matter in doses of 200 mg. per 100 cc. medium, it was possible to prepare from this phosphotungstic acid precipitate concentrates of the second accessory factor, 4 mg. of which, in the presence of 20 mg. of inositol per 100 cc. medium, gave the same growth. The material recovered from the phosphotungstic filtrates, though showing less activity per unit weight, still accounted for 25 to 30 % of the original active substance. The total recovery of factor II at this stage amounted to roughly 70 %, most of the remainder being lost with the inositol fraction.

About 300 g. of lentil concentrate were treated according to the above scheme in batches of 100 g. giving some 4 g. of the phosphotungstic precipitate fraction and 5 g. of the filtrate fraction. (In the following pages, it is to be understood that "phosphotungstic precipitate" and "phosphotungstic filtrate" refer to the fractions at this stage.)

(c) *Other chemical methods.* The further concentration of the active substance proved a matter of considerable difficulty. Attempts were made to prepare and fractionate crystalline picrates, but, although picrates of varying solubility were readily obtained, a considerable loss of activity was always found; this was not due, however, to the introduction of toxic substances into the fractions. Evidence was obtained that this inactivation was due to the mild oxidising action of the picric acid. In view of the reported stability of the substance towards oxidising agents, this seemed remarkable; however, the earlier experiments concerning oxidation were all made on the crude concentrates, and an apparent loss of stability with purer materials is not unknown in allied fields of research.

In another series of experiments, fractionation by means of copper salts was attempted [cf. Town, 1928]. From a small portion of the concentrate containing 250 unit doses of the second factor, the copper salts were prepared, and the following fractions separated: (i) insoluble in cold water, (ii) soluble in water, insoluble in methyl alcohol, (iii) soluble in water and in methyl alcohol, insoluble in absolute alcohol, (iv) soluble in absolute alcohol. In the growth tests on the fractions, after removal of Cu, fractions (i), (ii) and (iv) were found to be entirely inactive; fraction (i) was slightly toxic, possibly owing to the presence of traces of copper, but fractions (ii) and (iv) were free from toxicity. On the other hand, fraction (iii) was found to possess activity, but only to the extent of one-third that of the original concentrate fractionated. Thus there had evidently been a considerable loss of activity during the process, possibly owing to adsorption of the active substance on the copper sulphide precipitates. It may be recalled that Buston and Pramanik were unable to recover completely the activity of a concentrate which had been in contact with copper sulphide.

Fractionation of the lentil concentrate by physical methods.

(a) *Fractional electrolysis.* In view of the above recorded failures of chemical methods of fractionation, resort was had to methods of more physical nature. In the first place experiments were made using the method of fractional electrolysis, and evidence was obtained that the active substance tended to migrate towards the anode, while the greater part of the solids present moved towards the cathode. There was however a very considerable destruction of activity in the series of cells, and the method did not offer any prospect of giving more active concentrates.

(b) *Fractionation by solvents.* In a series of recent communications Block and Cowgill [1932] have studied the possibility of extracting vitamins of the B group from crude preparations by means of organic solvents and have claimed that they have obtained very considerable concentration under certain conditions. From the basic properties of the substances with which they were dealing Block and Cowgill argued that these substances should be soluble in organic solvents as bases and insoluble as salts.

Since in the case of the *Nematospora* factor it was impossible to state whether it was of acidic or basic nature, extractions with ether were carried out with both acid and alkaline solutions of the substance, solutions of the crude concentrate, without previous removal of the inositol, being used in the first place. An apparatus similar to that described by Wilson [1932] was employed; the ether used was previously purified carefully to remove traces of peroxides [see Garbarini, 1909]. The extracts and residues were in all cases freed from ether, acid *etc.*, and growth tests were made, such amounts of each fraction being used as would correspond to 0.2 g. of the lentil concentrate, on a basal medium con-

taining 20 mg./100 cc. of inositol, *i.e.* an adequate amount of inositol, irrespective of the amount present in the individual extracts. The results (Table V) showed that no extraction had taken place from the alkaline medium, indicating that it was unlikely that the substance was of basic nature. On the other hand, a certain amount of activity was removed by ether extraction from acid solution, although even here the greater part remained unextracted, and more prolonged treatment did not increase the proportion extracted.

Table V.

Fraction	Growth of mycelium (mg. dry weight)	
	Alkaline extraction	Acid extraction
Ether extract: 1 unit	Nil	41
2 units	Nil	91
Water phase: 1 unit	300	223
2 units	323	—
Toxicity test on ether extract	293	—

In other experiments, ether was replaced by butyl or amyl alcohol, the extractions being carried out in a faintly acid medium, using a continuous extraction apparatus modelled on that of Dakin [1920]; the extractions were usually carried out at reduced pressure, the temperature of the liquid being kept below 80°. Experiments with the crude concentrates again showed partial extraction of the active substance from acid solution, but further experiments using the more active concentrates obtained from the precipitate and filtrate fractions after the phosphotungstic acid treatment (p. 1862) showed that the active substance in these two fractions behaved somewhat differently towards the solvent.

(i) A 250 cc. portion of the filtrate fraction, containing 280 unit doses, was extracted in all for 18 hours with butyl alcohol (this solvent being preferred to amyl alcohol on account of its lower B.P.). On testing, it was found that the aqueous phase was completely inactive, showing that the active factor had passed completely into the butyl alcohol. The butyl alcohol itself, however, seemed to contain traces of some substance which checked the normal growth of the fungus, causing growth of the "staling" type, and a similar type of growth was observed even when the extract and residue were recombined. The mycelium, after growing normally at first, later gave irregular and sparse growth, so that the dry weights of mycelium obtained showed a distinct falling off of activity¹. The results of typical growth tests are given in Table VI.

Table VI.

Fraction	Growth of mycelium (mg. dry weight)
Aqueous phase after 12 hours	Nil
Alcoholic extract: 1 unit	146 (S)
2 units	242
Recombined	124 (S)

(S indicates staling growth.)

¹ Not all the samples of butyl alcohol used seemed to contain the substance producing "staling," for in at least one set of extractions normal growth was obtained (see Table VII, col. 3). It is understood that butyl alcohol is produced by a fermentation process and may contain traces of β -hydroxybutyric acid; this acid has been shown [Fuller, 1933] to possess bactericidal properties; it seems therefore not impossible that it may also affect the growth of other micro-organisms.

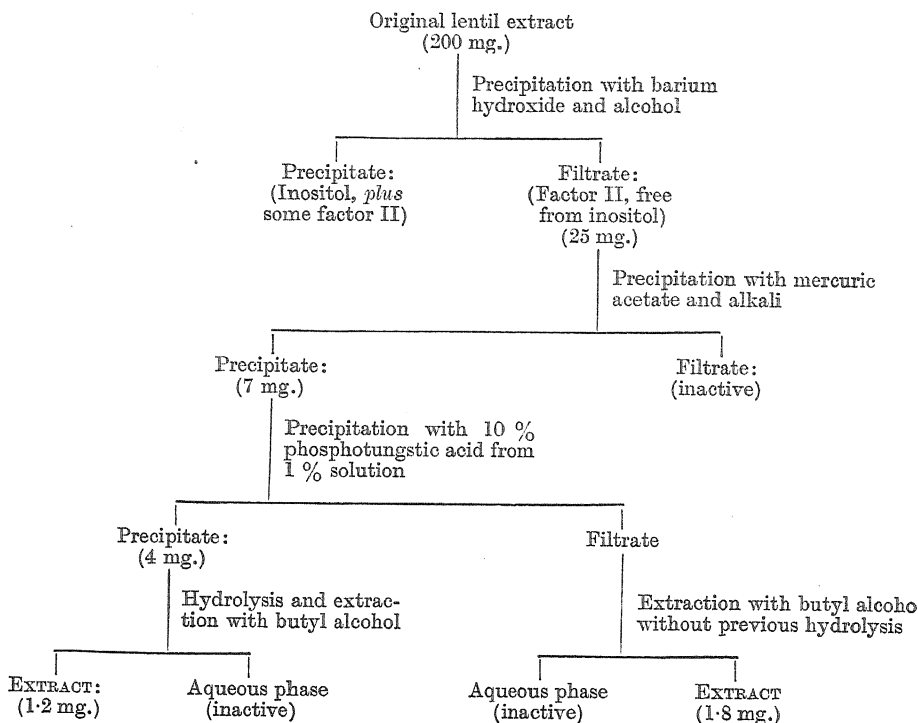
(ii) A portion of the concentrate from the phosphotungstic acid precipitate fraction was similarly extracted with butyl alcohol. After 18 hours' extraction, a growth test with 1 unit dose of the aqueous phase still gave 237 mg. (dry weight) of mycelium—roughly 85 % of the full growth, showing that but little of the activity had passed into the alcoholic solution. A further portion of the same concentrate was hydrolysed for 3 hours with 1 % sulphuric acid prior to extraction, and in this case the whole of the active substance passed rapidly into the alcoholic extract, the aqueous phase after 12 hours possessing no activity whatever. A further test was made on the filtrate fraction, after hydrolysing this in the same manner, but the treatment had no effect on the

Table VII.

Fraction	Growth of mycelium (mg. dry weight)		
	Filtrate hydrolysed	Precipitate hydrolysed	
		1	2
Aqueous phase after 6 hours	140	—	—
Aqueous phase after 12 hours	Nil	Nil	Nil
Alcoholic extract: 1 unit	253	204 (S)	280
2 units	255	—	—
Recombined	244	—	—

(S indicates staling growth.)

subsequent extraction (Table VII). The only explanation of the difference in behaviour of the active substance present in the phosphotungstic filtrate and precipitate fractions respectively seems to be that, in the latter, it forms part



of some more complex molecule, being probably combined with a basic substance, whereby it is rendered precipitable, whereas in the former it is present in an uncombined form, without basic properties. In either form, however, the substance is capable of exercising its growth-promoting function.

With regard to the activity of the extracts obtained after hydrolysis, that from the filtrate gave a growth of 255 mg. when supplied in a dose of 1.8 mg. per 100 cc. medium; for the precipitate fraction the dose was 1.2 mg. per 100 cc. Possibly the somewhat lower activity of the filtrate fraction was due to the presence therein of certain inactive substances, not present in the precipitate fraction, which were capable of extraction with butyl alcohol.

Further concentration of the active factor has not at the moment proceeded beyond this stage, except that some preliminary tests have shown that, from the precipitate fraction after hydrolysis, the whole of the bases are precipitated by phosphotungstic acid at p_H 2, and are quite inactive.

The steps in the concentration of the lentil extract are summarised in the accompanying diagram (see p. 1865); the figures quoted under each fraction represent the amount necessary, in 100 cc. of the medium, to produce the standard growth (270 mg.).

Reactions given by the active concentrates.

The observation that after hydrolysis the active factor was no longer precipitable by phosphotungstic acid, as well as the fact that in the uncombined form it escaped precipitation with this reagent in the first place (thus giving rise to the unexplained loss of 25–30 % of the activity in the phosphotungstic acid filtrate fraction) made it clear that the substance concerned was not a base. The most active concentrates still contained approximately 5 % N, on dry matter; the product obtained by butyl alcohol extraction of the filtrate fraction was richer in nitrogen (14.8 % N), although somewhat less active. Although this figure showed a considerable fall from the 18 % N recorded for the original phosphotungstic precipitate fraction, the possibility that activity was due to some nitrogenous substance was not excluded. In the combined form there seemed no doubt that the molecule contained a basic nitrogenous unit. The tests with the crude lentil concentrate (Table V) seemed to show that in the combined form the substance was insoluble in ether.

Some evidence seems to exist that the active substance is of acidic nature. Its extraction from acid solution only by ether; its behaviour under the influence of the electric current; its apparent ability to form copper salts and its non-precipitability in the free state by phosphotungstic acid point in this direction. It may be also that the persistent appearance of a portion of factor II in the inositol fraction was due to the formation of a barium salt by a part of the substance which was present in the lentil extract in the free state.

The most active concentrates were found to give a strong Molisch reaction, so that carbohydrates were still present. Pentoses were detected in the concentrates immediately before the butyl alcohol extraction, but the orcinol reaction was not given by the final concentrates. On the other hand, these concentrates gave a distinct naphthoresorcinol test for uronic acid, the test being indefinite in the case of the less active concentrates. It is interesting to recall that in the original paper of Farries and Bell [1930] it was suggested that the accessory factor was of the nature of a sugar acid; one class of sugar acids—the uronic acids—seem to be possessed of certain peculiar properties giving them a special influence on the course of metabolism. The stability of the substance here described towards heat and strong acids seems to preclude any possibility

of its being of the nature of a uronic acid, although the presence of a substance giving the naphthoresorcinol test is interesting. In any event, there is no suggestion that a state of purity has been approached; if it is possible to make any comparisons on the basis of the activity of the purest vitamin B preparations, at least 90 % of even the most active concentrate must be impurity, and there is no justification in attempting to assign the active factor to any chemical class.

Inorganic constituents of the concentrates.

Although Farries and Bell had already shown that the ash of their active "egg extract" was inactive in promoting growth of *N. gossypii*, the recent observations of Richards [1932] on the ability of certain metals, particularly thallium, to act as growth stimulants for yeasts, made it desirable to re-examine the question of the possibility of inorganic constituents of the lentil extracts acting as growth-promoting substances. Samples of the concentrate were examined spectrographically by Mr Hugh Ramage, to whom the authors' thanks are due. Apart from K, Na, Ca and Mg, the only element present in more than infinitesimal amount in the concentrate was nickel, which was present to an amount estimated at between 0.002 and 0.003 % of the crude concentrate. A series of platings was therefore made in order to discover whether nickel, in amounts of the order found in the concentrates, had any effect on the growth of the organism, but the results were entirely negative.

Tests with isomeric inositols.

In order to ascertain whether any of the naturally occurring isomerides of *i*-inositol, or related substances, could replace this body in the media, certain of these substances were prepared and their activity tested, in conjunction with factor II. Those used were: quercitol; quebrachitol; *l*-inositol, from quebrachitol; sennitol; *d*-inositol, from sennitol. In no case was any activity found.

SUMMARY.

1. The second factor necessary for the growth of *Nematospora gossypii* occurs in lentils in combination with an inactive nitrogenous base. It is readily liberated by gentle hydrolysis and exerts its growth-promoting influence both in the free and combined forms.
2. The second factor is also found associated with proteins, especially those of seeds, but probably does not form part of the protein molecule.
3. The active substance is precipitable by Neuberg and Kerb's mercuric acetate reagent, but (in the free form) not by phosphotungstic acid. In the combined form, the presence of a basic unit renders it precipitable by the latter reagent.
4. In the free form, the substance is extracted from acid solutions with organic solvents and seems to possess certain weakly acidic properties.
5. The most active concentrates prepared promote full growth when present to the extent of 1.2 mg. per 100 cc. medium, as compared with a dose of 200 mg. of the original lentil extract. Some reactions given by the concentrates are recorded and their possible significance is discussed.

We wish to record our indebtedness to Prof. W. Brown for facilities offered in the Department of Plant Pathology.

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CCLIV. THE ENZYMIC FORMATION OF HYDROGEN SULPHIDE BY CERTAIN HETEROTROPHIC BACTERIA.

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(Received November 1st, 1933.)

NONE of the numerous investigators who have studied the formation of hydrogen sulphide by bacteria has thoroughly analysed the relation between the organic sulphur-containing substrate employed and the formation of this compound. Furthermore, the failure to eliminate bacterial multiplication may have obscured to some extent the simple formation of hydrogen sulphide.

Sasaki and Otsuka [1912] found that most of a large number of bacteria studied by them formed hydrogen sulphide when cultivated in Fränkel's artificial medium to which either cystine or sulphur had been added. Certain of the strains investigated formed this gas from thiosulphate, only a few formed it from sulphite, and none produced it from taurine or from sulphate. Bürger [1914] found that the bacteria which he studied formed hydrogen sulphide from cystine but not from taurine. Tanner [1917], employing Fränkel's medium, studied a very large number of cultures and found that most of these formed hydrogen sulphide from peptone and from cystine, some from thiosulphate and thiourea, and that none of the strains formed this gas from 2-thiohydantoin, sulphite or sulphate. Almy and James [1926] showed that, when *P. vulgaris* was grown in a peptone solution containing added cystine, all the sulphur of this amino-acid could be recovered as hydrogen sulphide. Hydrogen sulphide was formed in cultures of this organism under both anaerobic and aerobic conditions. Tarr [1933] showed that washed cells of *P. vulgaris* decomposed cystine completely under anaerobic conditions, with the formation of two molecules each of hydrogen sulphide, ammonia, acetic and formic acids.

In the present investigation it has been shown that the process of hydrogen sulphide formation by washed cells of certain heterotrophic bacteria is enzymic in nature, a relatively high degree of specificity existing between the structure of the organic molecule attacked and the production of hydrogen sulphide. A study of certain well-known bacterial species has been made in order to determine the distribution of the enzyme concerned; and one factor which stimulates its formation in the bacterial cell has been found.

EXPERIMENTAL.

Many of the substances used, *viz.* glutathione (oxidised and reduced forms), cystine, cysteine hydrochloride, glycylcysteine, *N*-acetylcysteine, *N*-acetylcysteine, methyl and propylesters, *S*-ethylcysteine, *S*-benzylcysteine, methionine,

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glycylcysteine diketopiperazine, ergothioneine hydrochloride, 2-thio-4-methyl hydantoin and phthalimido-ethyl mercaptide were obtained through the kindness of Dr Pirie of this Department. All these compounds gave analytical figures in close agreement with those required by theory.

The sulphur compounds employed were dissolved in water to yield a concentration of 1 mg. or less per cc. The relatively insoluble compounds were dissolved in hot water. Where necessary the p_H of the solution was adjusted by titration to approximately 7.0 and the fluid sterilised by Seitz filtration. Cystine was dissolved in $N/100$ NaOH and the solution neutralised with sterile HCl after Seitz filtration. The sulphur used was autoclaved directly in the Thunberg tube employed.

Suspensions of *P. vulgaris* cells were prepared as described by Tarr [1933], with the exception that sterile Ringer solution was employed instead of sulphate solution. *Serratia marcescens* was cultivated for 36 hours at 28° on caseinogen digest agar, in other respects the technique employed in preparing cell suspensions of this organism was identical with that used in the case of *P. vulgaris*. Bacterial suspensions prepared in the above manner were stored aseptically at about 1° and were used within 5 days of preparation.

The following method was used in determining the formation of H_2S . 75 cc. Thunberg vacuum tubes, the necks of which were plugged with cotton-wool, were sterilised by autoclaving, the glass stoppers being wrapped and sterilised separately. 2 cc. of sterile $M/5$ phosphate buffer p_H 7.8, 5 cc. of bacterial suspension, and, except in control experiments, the required amount of substrate solution, were placed in each tube with aseptic precautions. The volume of liquid in each tube was made up to 20 cc. with sterile distilled water, the stopper, covered with sterile rubber grease, inserted and the tube evacuated. The evacuated tubes were incubated for 24 hours at 37° in experiments with *P. vulgaris* and at 28° in experiments with *S. marcescens*. All experiments were run in duplicate. At the conclusion of the incubation period the experimental fluid was acidified with 5 cc. of 5 % by volume H_2SO_4 and the H_2S aerated into 15 cc. of 2 % zinc acetate, suitable precautions being taken to avoid loss of the gas. The zinc acetate solution was employed for the colorimetric estimation of H_2S by the method of Almy [1925], care being taken to dissolve all traces of zinc sulphide in the acid dimethyl-*p*-phenylenediamine reagent.

In Table I the production of H_2S by *P. vulgaris* and *S. marcescens* cells from a large number of organic sulphur compounds is given. The results obtained permit the following conclusions to be drawn.

(1) Compounds containing the α -amino- β -thiolcarboxylic acid structure (cysteine), or its —S—S— form, yield over 75 % of their sulphur as H_2S .

(2) The substitution of the amino-group, carboxyl group, or both groups of cysteine inhibits the formation of H_2S enormously.

(3) The substitution of the sulphur of cysteine completely inhibits H_2S formation.

(4) α -Thiolcarboxylic-acids yield only very small amounts of H_2S .

(5) All the remaining organic sulphur compounds studied in which the S is not linked as in cysteine yield no H_2S .

It is concluded from the above data that H_2S formation from organic sulphur-containing substrates by the organisms investigated is specific inasmuch as, of the large number of compounds studied, only those which possess a cysteine or potential cysteine group are attacked to any significant extent.

Of the inorganic sulphur substrates studied sulphur was reduced to H_2S by both the bacteria, the comparative insolubility of this substance in water

Table I. *The anaerobic formation of H₂S by P. vulgaris and S. marcescens.*

Compound	Weight used in mg.	Theoretical amount H ₂ S mg.	H ₂ S recovered			
			<i>P. vulgaris</i>		<i>S. marcescens</i>	
			mg.	%*	mg.	%*
Control†	0.0	0.0	0.004‡	—	0.004	—
			0.004‡		0.004	
Cysteine	0.5	0.141	0.112‡	79	0.120	80
			0.120‡		0.115	
Cystine	0.5	0.142	0.120‡	83	0.120	80
			0.123‡		0.115	
Glutathione (reduced form)	1.0	0.111	0.095‡	82	0.091	78
			0.095		0.091	
Glutathione (oxidised form)	1.0	0.111	0.098‡	82	0.090	79
			0.092		0.094	
Glycylcysteine	0.5	0.095	0.080	82	0.074	76
			0.084		0.078	
N-Acetylcysteine	0.5	0.104	0.020	15	0.006	2
			0.020		0.006	
N-Acetylcysteine methyl ester	0.5	0.096	0.010	6	0.006	2
			0.010		0.006	
N-Acetylcysteine propyl ester	0.5	0.083	0.006	2	0.017	16
			0.006		0.017	
Thiolacetic acid	0.25	0.093	0.006	2	0.010	6
			0.006		0.009	
α-Thiolpropionic acid	0.5	0.159	0.006	2	0.004§	0
			0.006		0.004	
S-Ethylcysteine	0.5	0.114	0.004‡	0	0.004	0
			0.004		0.004	
S-Benzylcysteine	0.5	0.086	0.004‡	0	0.004	0
			0.004		0.004	
Methionine	0.5	0.114	0.004‡	0	0.004	0
			0.004		0.004	
Glycylcysteine diketopiperazine	0.5	0.089	0.004	0	0.004	0
			0.004		0.004	
Ergothioneine	1.0	0.113	0.002	0	0.002	0
			0.002		0.002	
Phthalimido-ethyl mercaptide	0.5	0.093	0.004	0	0.004	0
			0.004		0.004	
Thiourea	0.25	0.112	0.004‡	0	0.004	0
			0.004		0.004	
Monophenylthiourea	0.5	0.112	0.004‡	0	0.004	0
			0.004		0.004	
Allylthiourea	0.5	0.146	0.002	0	0.004	0
			0.002		0.004	
2-Thio-4-methylhydantoin	0.5	0.131	0.002	0	0.004	0
			0.002		0.004	
Sodium thiosulphate	0.5	0.108	0.087	78	0.004§	0
			0.087		0.004	
Sodium sulphite	0.5	0.135	0.004	0	0.004§	0
			0.004		0.004	
Sodium sulphate	0.5	0.120	0.004	0	0.004§	0
			0.004		0.004	
Sulphur	1.0	1.062	0.026	2	0.029§	2
			0.029		0.025	

* Calculated on the theoretical value after subtracting the control.

† No appreciable variation was found in the control values for different suspensions of the same organism.

‡ 31 mg. dry weight of bacteria per exp. Exps. in the same column without asterisk had 34 mg. dry weight per exp.

§ 66 mg. dry weight of bacteria per exp. Exps. in the same column without asterisk had 49 mg. dry weight per exp.

probably limiting the amount reduced. Neither sulphite nor sulphate was reduced. The fact that thiosulphate was strongly attacked with the formation of H_2S by *P. vulgaris* and was not attacked by *S. marcescens*, and that cysteine was attacked by both organisms, suggests that H_2S formation from thiosulphate and from cysteine is due to two distinct mechanisms.

Distribution of the enzyme forming H_2S from cysteine.

In order to determine the distribution of the enzyme among different bacterial species a study was made of aerobic, facultative anaerobic and anaerobic bacteria. Washed cell suspensions of *B. subtilis*, *B. megatherium*, *Ps. aeruginosa*, *E. coli* and *A. faecalis* were prepared by the method used in obtaining *P. vulgaris* cells. *S. lutea* was cultivated for 48 hours at 28° , the technique of preparing cell suspensions of this organism being in other respects identical with that used in preparing *P. vulgaris* cells. *C. sporogenes* was grown anaerobically for 44 hours on caseinogen digest broth, the cells being washed and suspended in Ringer solution by the method already referred to. Duplicate experiments for determining the amount of H_2S produced from 0.5 mg. of cystine, together with the corresponding controls, were run for all the organisms studied, the technique employed being identical with that already described. The experimental solu-

Table II. *The anaerobic formation of H_2S from 0.5 mg. of cystine by different bacteria.*

Organism	Dry wt. of bacteria per experiment mg.	Experiment	H ₂ S recovered (theory for 0.5 mg. cystine = 0.142 mg.)	
			mg.	% of theoretical
<i>A. faecalis</i>	26	Controls	0.002 0.002	8
		Cystine	0.013 0.013	
<i>B. subtilis</i>	23	Controls	0.000 0.000	11
		Cystine	0.018 0.014	
<i>B. megatherium</i>	16	Controls	0.000 0.000	1
		Cystine	0.002 0.002	
<i>E. coli</i>	21	Controls	0.002 0.002	78
		Cystine	0.111 0.111	
<i>Ps. aeruginosa</i>	33	Controls	0.004 0.004	6
		Cystine	0.012 0.014	
<i>S. lutea</i>	55	Controls	0.000 0.001	0
		Cystine	0.001 0.001	
<i>C. sporogenes</i>	4	Controls	0.004 0.004	43
		Cystine	0.065 0.065	

tions were incubated for 24 hours, at 28° in the case of *S. lutea*, and at 37° in the case of all other bacteria studied.

The results of these experiments are given in Table II. From this Table it is evident that the power of various bacteria to produce H_2S from cysteine varies considerably. Thus *S. lutea* and *B. megatherium* are inactive or nearly so, while *E. coli* and *C. sporogenes* are very active. It is interesting to note that the enzyme is present both in cells of such aerobic bacteria as *B. subtilis* and *A. faecalis*, and in the strict anaerobe *C. sporogenes*.

The velocity of H_2S formation from cysteine by washed cells of P. vulgaris obtained from an identical medium with and without added cysteine.

P. vulgaris was grown in the usual manner on caseinogen digest agar to which 0.1 % of cysteine (as the neutral hydrochloride solution sterilised by Seitz filtration) had been added prior to solidification of the medium, and on an identical medium without added cysteine. Washed cell suspensions of both these types of bacteria prepared as usual were stored at 1° and were used within 12 hours of preparation. H_2S formation from 0.5 mg. of cysteine was determined as already described, a number of experiments being run in the case of each suspension. The velocity of H_2S formation was determined by incubating the Thunberg tubes in a water-bath at 37° and withdrawing them at suitable intervals for the estimation of this gas. In Fig. 1 the results of these experi-

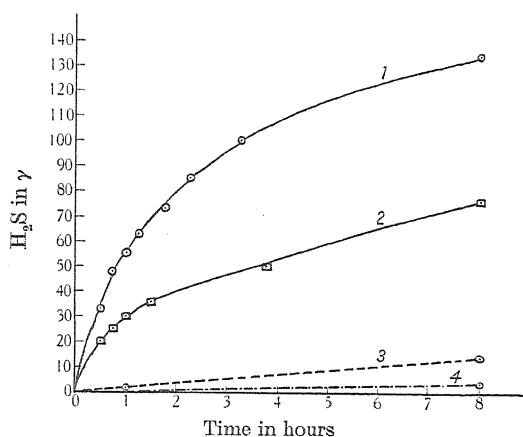


Fig. 1. 1. \circ — \circ H_2S formed from cysteine by *P. vulgaris* cells obtained from medium with 0.1 % added cysteine; 2. \square — \square H_2S formed from cysteine by *P. vulgaris* cells obtained from medium without added cysteine; 3. — — — H_2S formed in absence of cysteine by *P. vulgaris* cells obtained from medium with 0.1 % added cysteine; 4. — — — — H_2S formed in absence of cysteine by *P. vulgaris* cells obtained from medium without added cysteine.

1 and 3. 14 mg. dry wt. of bacteria per exp. 2 and 4. 24 mg. dry wt. of bacteria per exp.

ments are plotted graphically. It is clear from this diagram that H_2S is formed with much greater rapidity by cells cultivated in the presence of 0.1 % cysteine than by cells cultivated on an identical medium in the absence of added cysteine. Suspensions of cells cultivated in the medium rich in cysteine also give off more H_2S when incubated in the absence of cysteine than do suspensions obtained from the medium without added cysteine. The formation of H_2S from cysteine takes place with greatest velocity at the commencement of the experiment. After 8 hours 85 % of the cysteine added was recovered as H_2S in the

experiment in which cells from the medium rich in cysteine were employed, while in the case of the other suspension only 51 % of the cysteine sulphur was recovered as H_2S . Calculation showed that, in this experiment, 10 mg. (dry weight) of bacteria from the medium rich in cysteine would be capable of forming 85% of H_2S in 8 hours, while, under similar experimental conditions, 10 mg. (dry weight) of bacterial cells from the medium with no added cysteine would only form 38% of H_2S in 8 hours.

SUMMARY.

1. The formation of hydrogen sulphide from a variety of organic and inorganic sulphur compounds has been studied employing washed cells of *P. vulgaris* and *S. marcescens*.
2. Of the organic sulphur compounds studied only cysteine, cystine or those containing either of these molecules yield over 75 % of their sulphur as H_2S .
3. Substituted cysteine compounds and α -thiolcarboxylic acids, when attacked, only yield very small amounts of H_2S , and all other organic sulphur compounds studied yield no H_2S .
4. Of the inorganic sulphur compounds studied sulphur is reduced to H_2S by both bacteria, while neither sulphite nor sulphate is reduced with the formation of H_2S by either strain.
5. Thiosulphate is reduced with the formation of H_2S by *P. vulgaris*, but not by *S. marcescens*.
6. The formation of H_2S from organic sulphur compounds appears, therefore, to require the presence of a specific enzyme in the bacterial cell.
7. This enzyme has not been found in the cells of all bacteria studied, but has been found in the cells of aerobes, facultative anaerobes and in one strict anaerobe.
8. Cysteine added to the medium employed for producing the bacterial cells stimulates the formation of the enzyme.

This investigation was largely made possible by the kindness of Dr N. W. Pirie of this Department in supplying me with most of the organic sulphur compounds employed. My thanks are due to Dr Spooner of the Department of Pathology for giving me certain of the cultures used. To Sir F. G. Hopkins I am indebted for his constant interest and encouragement.

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CCLV. THE NORMAL SERUM-CALCIUM AND MAGNESIUM OF THE RAT: THEIR RELATION TO SEX AND AGE.

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(Received November 1st, 1933.)

THE normal serum-magnesium of the rat has not hitherto been determined, except perhaps for a very few animals; some figures given by Watchorn [1932] for instance, were too few to be of value. The present paper gives the data from approximately 100 rats. The opportunity was taken to study the calcium also, for although the rat has frequently been used for serum-calcium studies (notably in work on irradiated ergosterol) the normal has received but little attention, a few animals only having generally been used. The work of Cameron referred to later is, however, an exception to this general statement.

EXPERIMENTAL.

The rats (black and white piebalds) when weaned were placed on a diet consisting of "light white casein" 23, rice starch 40, cane sugar 20, arachis oil 12, salts (modified McCollum and Davis, for details see Watchorn [1932]) 5, dried yeast 10, cod-liver oil 2.5. Fresh water was provided *ad lib.*, and in addition fresh milk was given several times a week. The percentage of magnesium in the basal diet was 0.035, which is within the range suggested by Lavollay [1931] as most suitable for growth. The males and females were kept separate throughout, and all animals were housed in large cages giving ample room for exercise. The room was kept at a constant temperature of 70° F. The rats were always killed in the early afternoon and the stomach was invariably empty, no fresh food having been given that morning. Blood was removed by heart puncture under chloroform anaesthesia. All females were taken in dioestrus, and each rat was examined for evidence of disease; if such was present the blood was not used. Any haemoglobin-stained sera were also discarded.

Calcium was estimated by Clark and Collip's [1925] modification of Kramer and Tisdall's method, and magnesium by the Bell-Doisy colorimetric determination of the precipitated magnesium ammonium phosphate. Owing to the high concentration of magnesium in the serum of the rat it is possible to make accurate determinations on 1 cc. of serum. 2 cc. were, however, taken whenever possible on account of the accompanying calcium determination.

Results.

The rats of each sex have been divided into two groups, "young" and "adult." The former were all 3-4 months old, and the latter ranged from 8½ to 16 months. Originally the adults were subdivided into those under one

year and those over one year, but as no difference could be detected in the few 16-months old rats available, they have finally been grouped together with the others. The results are given in Tables I and II.

About 20 rats reared on a mixed stock diet of natural foodstuffs have also been examined. The values for serum-calcium and magnesium all came within the normal limits given in the following tables.

Table I. *Normal rat serum-calcium.*

No. of animals	(mg. per 100 cc.)			
	Males		Females	
	Young 28	Adult 27	Young 24	Adult 24
Range of variation	10.70-13.79	10.29-13.16	11.18-13.23	9.61-14.04
Mean value	12.23	11.92	12.00	11.60
Standard deviation of the mean	± 0.14	± 0.16	± 0.12	± 0.26
Standard deviation of the series	0.75	0.84	0.61	1.29

Table II. *Normal rat serum-magnesium.*

No. of animals	(mg. per 100 cc.)			
	Males		Females	
	Young 28	Adult 25	Young 23	Adult 23
Range of variation	4.01-6.89	3.30-5.24	3.71-5.10	3.63-5.69
Mean value	5.36	4.43	4.39	4.43
Standard deviation of the mean	± 0.12	± 0.10	± 0.08	± 0.10
Standard deviation of the series	0.64	0.49	0.39	0.50

DISCUSSION.

Calcium. From a much smaller series Parhon and Werner [1932] found that the normal serum-calcium of the rat varied from 11.0 to 13.5 mg. per 100 cc., with an average of 12.2 mg. Tweedy and Chandler [1929] give a range of 9.25-12.5 mg. per 100 cc., and Hess *et al.* [1932] in a series of 11 normal young rats have values from 10.1 to 13.0 mg. per 100 cc., the average being 11.18 mg. The figures in Table I are of the same order as those just quoted, as also are those given by Dixon [1933]. On the other hand, Hess *et al.* in an earlier paper [1928-29] state that the rat's normal serum-calcium is 10.0 mg. per 100 cc., and Harris and Stewart [1929] that the range is 9.5-10.5 mg., though in neither case are figures given or authorities quoted. The "hypercalcaemic" figure found by Harris and Stewart seems in fact to be well within the normal range.

In the present series the serum-calcium level of both young and adult females was slightly lower than that of the corresponding males. The adults of both sexes had lower average values than the young. The question arises as to whether these small differences can be regarded as significant. If the animals are grouped into "young" and "adult" irrespective of sex and the results treated statistically, the following figures are obtained:

Mean value of all young = 12.16 mg. per 100 cc.
 Mean value of all adult = 11.77 mg. per 100 cc.
 Difference = 0.39 mg. per 100 cc.

The value of *t* is 2.171, *P* lies between 0.05 and 0.02, and thus the difference due to age is significant. Parhon and Werner [1932] noticed a tendency for

serum-calcium to decrease with age in many species, including the rat. Cameron [1928] found the contrary.

Males and females grouped irrespectively of age give the following figures:

Mean value of all males = 12.10 mg. per 100 cc.

Mean value of all females = 11.80 mg. per 100 cc.

Difference = 0.30 mg. per 100 cc.

The value of t is 1.708, so that P lies between 0.1 and 0.05; from this it is impossible to say with certainty whether the difference between sexes is significant or not. McIsaac [1928] found slightly lower concentrations of calcium in the serum of young female rabbits compared with males of similar ages; this sex difference became more marked with age. Meglitzky [1927] found a similar sex difference in cats, and Boynton and Greisheimer [1930-31] for men and women. Charles [1931] found an average of 11.96 mg. per 100 cc. for 12 male rabbits and 11.51 mg. for 12 females—though again, statistically, the difference is not significant.

Boynton and Greisheimer [1930-31] noticed that men showed a smaller range of serum-calcium variation than women, and Okey *et al.* [1930] state that the day to day variations for men are smaller than for women. It is apparent from Table I that the serum-calcium of the adult female rat is more variable than that of the adult male or the young of either sex.

Cameron and Williamson [1927] give the average winter and summer serum-calcium value for the albino rat as 10.4 mg. per 100 cc., with a range of 8.3-12.1 mg. Values in the spring were definitely lower. Cameron [1928] further investigated the matter and found that the younger the rat the more variable was the serum-calcium and the more liable to be low in concentration. The curve for blood-calcium values appeared to be parallel with the curve for solar ultra-violet radiation, and Cameron suggests that the low levels frequently found in his young rats were the result of insufficient and seasonal variations in the vitamin D content of the foodstuffs fed. Cameron's figures were obtained from the study of a large number of rats and their accuracy is not to be doubted; nevertheless they cannot be taken as representing the normal picture for the rat living in this country under standard conditions, where the vitamin intake is unaffected by seasonal changes.

Magnesium. The serum-magnesium of the rat is approximately double that of man. The average value of the females did not change with age and was the same as for adult males. The young males, however, had definitely higher values. The difference between the average values of young and adult males was 0.93 mg. per 100 cc. The value of t is 5.856, whence P is less than 0.01 and the difference definitely significant. Had all the animals been examined irrespectively of age, there would still have appeared to be a difference between the males and females of 0.51 mg. per 100 cc., and this again is statistically significant ($t=4.907$ and P is less than 0.01). Charles [1931] found slightly lower serum-magnesium values in female rabbits compared with males, but the difference was not statistically significant for the number of animals used.

The lower serum-magnesium level of the young female rats is interesting in connection with magnesium deficiency. It has been the experience of the writer that females receiving a magnesium-deficient diet show pathological symptoms much more rapidly than do the males of the same age.

Correlation of serum-calcium and magnesium. Brookfield [1933] stated that there is an inverse relationship between the serum-calcium and magnesium of rabbits. No such inverse relationship was apparent from an inspection of the

figures given above for rats. An examination of the average values, on the contrary, indicates a tendency for these two serum constituents to vary in the same direction. The correlation coefficient of the 97 pairs of figures available for treatment is +0.36 and *P* is less than 0.01. There thus appears to be a definite, though not very rigid, relation between the serum-calcium and magnesium, but the coefficient being positive the ratio of the two is direct and not inverse.

SUMMARY.

1. The serum-calcium and magnesium values of approximately 100 rats have been determined.
2. Young rats had a slightly, but probably significantly, higher serum-calcium level than adult animals.
3. The females had a slightly lower serum-calcium than the males. The difference was not definitely significant.
4. The average serum-magnesium of adult males and of young and adult females was the same (4.4 mg. per 100 cc.), but young males had a significantly higher average (5.36 mg. per 100 cc.).
5. There was no seasonal variation in either mineral with the diet used.

The writer is indebted to the Medical Research Council for a full time personal grant.

She also wishes to thank Mr Wishart of the School of Agriculture for advice and encouragement concerning the statistics.

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CCLVI. THE PROTEINS OF GRASSES.

II. A NEW METHOD OF PREPARATION.

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(Received November 1st, 1933.)

IN the preliminary paper of this series [Miller and Chibnall, 1932], it was pointed out that the original ether method of Chibnall [1923] for the preparation of leaf proteins gave negligible amounts of protein when applied to various grasses, and a modification involving the use of ether-water in lieu of ether was described which in certain cases had enabled us to prepare the protein from cocksfoot. The yield of protein obtained however varied greatly and in most cases was disappointingly small. Many experiments were made to find out the inherent weakness of the method employed, and one of them (Exp. 4) described in the preliminary paper suggested that the ultimate yield of protein could be enhanced two- or three-fold if the grass, instead of being treated as soon as possible after cutting, was first of all set aside for about 8 hours, as though some change affecting the "aggregation" of the leaf proteins took place during this short period, thereby enabling them to disperse more readily into colloidal solution at the appropriate stage in the preparation. At the same time it was clearly pointed out that the influence of ether or ether-water, together with a "time factor," on the cytolysis of the leaf cell protoplasts—the first and essential step in the preparation of the leaf proteins—was not yet clearly understood, for although the use of ether-water and a "time factor" of 8 hours gave fair yields of protein from grasses cut in spring, they gave, curiously enough as it seemed at the time, negligible yields of protein from grasses cut from the same plots the following autumn.

Further experiments carried out during the last 18 months have indeed shown that our former conclusions were quite erroneous. Ether-water has been used as a cytolysing agent in the preparation of proteins in excellent yield from a large number of grasses and other forage crops, and we now know that the essential point in its employment is that it must have been used at least once before to cytolysise an appropriate amount of grass. This subtle difference in behaviour between what we shall henceforth refer to for convenience as "fresh" and "used" ether-water was quite unlooked for, because our previous experience in the use of ether itself as the cytolysing agent for leaves such as spinach or runner bean had taught us to look upon the ether content of the ether-water as the potent factor in its use. When fresh ether-water is used to cytolysise leaves it not only becomes contaminated with sap which exudes from the leaf cells but it also loses ether by evaporation during the subsequent handling of the leaf material. For this reason it had been customary in our laboratory to employ a new sample of ether-water with each portion of grass undergoing treatment. Occasionally, however, a shortage of ether compelled us to use the ether-water a

second or third time, and it is a coincidence that this occurred when a sample of the grass had been deliberately set aside for 8 hours. The greatly increased yield of protein then obtained was naturally, but we now know wrongly, ascribed to the "time factor" mentioned above. Further investigation very soon disclosed the nature of the extraordinary difference in action between "fresh" and "used" ether-water and since we now believe that this slight modification in technique permits the preparation of proteins in good yield not only from leaves of herbaceous plants which were amenable to the earlier ether treatment but also from leaves of ligneous plants from which it has hitherto been impossible to prepare proteins at all, it is necessary to describe the cytolytic action in some detail.

When cut grass is immersed in "fresh" ether-water for ten minutes the latter slowly turns brown. The leaf cells are cytolysed and the protoplasts become freely permeable to the aqueous contents of the vacuoles; consequently those constituents of the cell whose molecules are sufficiently small will diffuse freely through the cellulose wall into the ether-water. If a section of this cytolysed grass be cut the cells present the same appearances as those of spinach leaves cytolysed with ether [Chibnall, 1923]. In each cell the protoplast has collapsed and has shrunk to one end, the rest of the cell being filled with a brown water-clear liquid which is the vacuole fluid diluted with ether-water.

If a fresh batch of cut grass is now immersed in this "used" ether-water, cytolysis of the cells takes place to all appearances as readily as before, and the ether-water becomes further contaminated and more darkly coloured. Two striking differences can, however, be readily observed. In the first place there is a relatively enormous uptake of "used" ether-water by the cells, presumably before the semi-permeability of the protoplasts has been completely destroyed. In the second place a cut section shows that there is no evidence of collapse or shrinkage of the protoplasts, although these have been rendered freely permeable. The section, in fact, differs but little from that of a fresh untreated blade of grass, in which the turgid protoplasts fill the whole interior of the cell.

It is not possible at the present time to give a physiological explanation of the difference in behaviour between the "fresh" and "used" ether-water, but we believe that the following account affords a reasonable explanation of certain of the observed facts which bear on the yield of protein ultimately obtained. When the cut grass is immersed in "fresh" ether-water, the semi-permeability of the protoplast is destroyed extremely rapidly, so that the fluid of the vacuole, which contains a high concentration of solutes, is suddenly released. This fluid, as it diffuses out through the now freely permeable protoplast, causes partial dehydration or perhaps partial denaturation of the colloidal proteins which are one of the chief components of the cytoplasm, and may thus help to bring about the collapse and shrinkage of the protoplast. At the stage in the preparation of the proteins therefore when the pressed and washed leaf residues are ground up with water the cytoplasmic contents of the cells are not readily dispersed into colloidal solution. When "used" ether-water is employed the cytolysis of the cells is brought about in a modified way. The anaesthetic action of the ether is depressed, so that instead of causing the almost instantaneous death of the cell there is in the earlier stage only a slow decrease in semi-permeability. In some way which it is not yet possible to explain the response of the cells is changed, with the surprising result that water passes rapidly through the protoplast into the vacuole. The ether anaesthesia ultimately renders the protoplast freely permeable, but the vacuole fluid has been by now so diluted with ether-water that when it diffuses out through the protoplast the colloidal proteins suffer no

appreciable dehydration or denaturation, so that not only is the protoplast left, to external appearances, unchanged, but at the stage in the preparation of the leaf proteins referred to above the cytoplasmic contents of the cells are readily dispersed into colloidal solution. Appropriate experiments have shown that this modified action of "used" ether-water is due not to the lowered concentration of ether but to the substances which diffuse out from the cytolyzed cells, and ether-water which has been used initially with one particular species of grass will act as "used" ether-water for any other species.

If one accepts the validity of the above explanation, it appears that the high yields of protein obtained from spinach and other leaves by the older ether-method, which caused the collapse and shrinkage of the protoplasts, were due to the much lower concentration of solutes initially present in these leaf cell vacuoles.

EXPERIMENTAL.

Materials used. The grasses used in the present research were pure strains taken from specially cultivated plots which were sown in the autumn of 1931.

Table I. *Details of proteins prepared from various grasses.*

Species	Date of sampling	Sample of leaf material				Extracted protein		
		Fresh weight kg.	Dry weight %	Total N %	Total protein-N %	Total weight g.	N (Ash-free) %	Yield in % of total leaf protein
Cocksfoot (<i>Dactylis glomerata</i>) (Batch Q)	20. v. 32	8.0	14.5	6.4	5.3	73	14.1	16.8
" " (" R)	27. v. 32	17.0	14.1	6.5	5.0	271	13.6	30.9
" " (" S)	3. vi. 32	20.0	13.0	6.8	5.7	245	14.0	23.3
" " (" U)	16. vi. 32	16.0	17.5	5.8	5.1	216	13.0	19.8
" " (" AB)	29. v. 33	30.0	13.0	6.15	5.4	397	14.6	27.5
" " (" AC)	7. vi. 33	16.0	15.0	6.0	5.3	210	13.4	22.4
" " (" AD)	22. vi. 33	30.6	17.8	5.65	4.9	360	13.2	17.9
" " (" AE)	11. ix. 33	7.5	18.3	6.07	5.5	97	13.3	17.5
Rough-stalked meadow grass (<i>Poa trivialis</i>)	4. x. 32	2.9	18.0	5.6	4.9	26	13.4	13.6
" "	27. vi. 33	12.0	14.0	6.2	5.3	89	13.8	13.7
Timothy (<i>Phleum pratense</i>)	18. x. 32	0.9	27.0	4.7	4.1	7	13.8	9.7
Chewings fescue (<i>Festuca rubra</i> var. <i>fallax</i> (Hack))	26. ix. 32	7.5	16.0	6.15	4.8	73	14.1	17.8
Hard fescue (<i>Festuca duriuscula</i>)	29. ix. 32	4.0	22.5	6.80	5.5	37	15.0	11.1
" "	29. vi. 33	8.0	19.0	5.95	4.9	65	14.6	12.8
Red fescue (<i>Festuca rubra</i>)	29. ix. 32	2.0	21.0	6.15	5.0	27	14.4	18.4
" "	29. vi. 32	8.0	16.0	5.85	4.8	77	14.2	17.7
Tall fescue (<i>Festuca elatior</i>)	11. x. 32	1.4	16.6	5.75	5.0	13	13.7	15.4
" "	26. vi. 33	4.0	17.0	5.8	4.9	40	13.6	16.3
Meadow fescue (<i>Festuca pratensis</i>)	18. x. 32	0.7	32.0	3.4	2.9	7	13.8	14.7
" "	27. vi. 33	6.0	15.0	5.7	4.8	(6)	13.9	—
Italian ryegrass (<i>Lolium italicum</i>)	11. x. 32	1.7	13.7	6.3	5.3	13	14.0	14.8
" "	26. vi. 33	4.0	15.0	5.9	5.2	45	14.1	20.5
Perennial ryegrass (<i>Lolium perenne</i>)	6. xi. 33	7.0	18.1	4.6	3.8	82	12.8	21.8
Crested dog's tail (<i>Cyanosurus cristatus</i>)	18. x. 32	1.1	25.3	4.5	3.7	14	13.8	18.6
" "	27. vi. 33	6.0	16.0	5.25	4.5	46	14.9	16.1
*Bent (<i>Agrostis</i> sp.)	25. x. 33	4.0	27.0	2.43	2.1	32	10.3	14.6
*Yorkshire fog (<i>Holcus lanatus</i>)	23. x. 33	2.3	20.2	3.26	—	4.1	11.8	—
*Wild white clover (<i>Trifolium repens</i>)	18. x. 33	26.4	13.6	5.09	4.3	319	13.2	28.0
*Red clover (<i>Trifolium pratense</i>)	23. x. 33	9.3	23.7	2.71	2.4	101	12.8	24.5
*Lucerne (<i>Medicago sativa</i>)	29. vi. 33	12.0	21.0	3.25	2.8	61	14.4	12.6
*Yarrow (<i>Achillea Millefolium</i>)	24. x. 33	10.0	10.0	3.92	3.4	26	10.0	7.6

* From plots which had not received a dressing of ammonium sulphate.

In the spring of 1932 and 1933 each plot received a heavy dressing of complete fertiliser, and to obtain the maximum amount of protein from a given quantity of grass the plots were heavily treated at appropriate intervals with 3 cwt. of ammonium sulphate per acre. About 8-10 days before the grass material was required the plots were closely cut back with a mowing machine and then dressed with the fertiliser. In the absence of rain the plots were watered daily, and when cut with the mower to provide the experimental material the blades stood 3-5 inches high. In the case of cocksfoot, the protein of which we are using for amino-acid analysis and therefore require in large amount, a second cutting was taken about 7 days later. After growing for another week the plots were again cut back (grass discarded) and treated as before with ammonium sulphate, when they were again ready to give two high-nitrogen crops at the time intervals mentioned above. Details of the samples used are given in Table I. For convenience the total protein-N was determined by the conventional method of Stutzer. As one of the ultimate objects of the present research on grasses is to determine the nutritive value of forage crops we have also prepared proteins from lucerne, red and white clover and yarrow. All the proteins described in Table I were prepared by a standard method founded on the principles discussed above, and the practical details will be best illustrated by describing a typical preparation of cocksfoot protein in some detail.

Preparation of protein from 20 kg. of freshly cut cocksfoot.

Some beds of cocksfoot at the Imperial College Field Station at Slough were cut back with a mowing machine on May 20th, 1932, and then dressed with 3 cwt. of ammonium sulphate per acre. A first cutting (Batch R) was taken on May 27th and a second (Batch S), with which the present experiment is concerned, on June 3rd at 7 a.m. There was no appreciable dew, and the total fresh weight was 22 kg. the dry weight 13.0 % and the N 6.8 % of the dry weight.

A sample of 2 kg. was immersed in 5 litres of "fresh" ether-water contained in a deep enamelled pan, and at the end of 10 minutes the pan was tilted so that the resulting brown liquid could be drained off from the grass. The volume of "used" ether-water thus collected was 4500 cc., the remaining 500 cc. being retained on the surface of the grass. In many of the experiments not recorded in detail in this paper the batch of grass had been cut early in the morning following a rainy night. In such cases the surface of the grass was already wet, and when a sample was treated with "fresh" ether-water as described above there was no loss on draining, showing that the loss of 500 cc. in the present case was due to the "wetting" of the surface of the blades. The cytolysed grass was next enclosed in thick filter-cloth, which was placed in the steel cylinder 11 inches high and of 6 inches internal diameter belonging to a Buchner press. A well-fitting plunger was used to apply the maximum pressure for 4 minutes. The volume of expressed juice was 1400 cc. and on removal from the press the cylindrical cake of compressed grass was 9 cm. high. Nothing further was done with this sample, which had been worked up merely to provide the necessary "used" ether-water for the preparation of protein from the remaining 20 kg. of grass.

Another sample of 2 kg. was next immersed for 10 minutes in this 4500 cc. of "used" ether-water. On draining off the liquid only 3100 cc. was collected, so that if we assume that 500 cc. were required to "wet" the grass the remaining 900 cc. must have been actually taken up by the 2 kg. of grass. This enormous

intake of water by grass cytolysed with "used" ether-water has been commented on at some length above. The sample of grass was next enveloped in filter-cloth and pressed for 4 minutes as before. On removal from the press the cylindrical cake of grass residue did not remain compressed as in the previous sample, but the blades of grass separated slightly so that the height of the cake was 16 cm. as against 9 cm., again emphasising the fact that "used" ether-water had brought about cytolysis with less internal breakdown in the leaf-cells than was the case with "fresh" ether-water. The residue was allowed to imbibe water for 4 minutes and pressed as before. This operation was repeated twice more in order to wash away the easily diffusible contents of the cells. The final leaf residue was then ground to a pulp in a meat chopper with 3 litres of water, and the débris of cell wall material removed by squeezing through silk gauze. This débris was again treated in a similar way with a further 2 litres of water, and the two green colloidal extracts thus obtained were filtered with very slight suction on a 24 cm. Büchner funnel through a well-rammed pad of paper-pulp about 6-7 cm. thick. A clear brown protein filtrate was thus obtained.

Meanwhile a third 2 kg. sample of grass was immersed in the 3100 cc. of "used" ether-water given by the previous sample, 1900 cc. of press-juice being added to bring the total volume to 5000 cc. The remaining eight 2 kg. samples of grass were also treated in a similar way at such time intervals that there was always sufficient green colloidal extract to permit of continuous filtration through two of the paper-pulp pads. In each case the volume of "used" ether-water was made up to 5000 cc. with press-juice, and before the treatment of the 7th sample of grass it was reinforced by shaking with 100 cc. of ether.

The volume of the final filtrate was 47.3 litres, and 580 cc. of 2.04 *N* HCl were required to precipitate the protein at its isoelectric point. After standing overnight the supernatant liquid was syphoned off and the protein coagulated by heating on a water-bath. It filtered readily at the pump, and was purified by extracting once with boiling water, then boiling 5 times successively with 95 % alcohol and finally once with absolute alcohol. The weight of the moisture-free protein was 245 g., and it contained 13.9 % of N and 0.9 % of ash. The N, ash-free, was 14.0 %. As only 20 kg. of the batch of grass had been used to prepare the protein this yield represents 9.3 % of the total dry weight of the grass, 19.1 % of the total grass-N and 23.0 % of the total protein-N. These figures are twice as great as those obtained from spinach by the original ether method of preparation [Chibnall, 1924] and from cocksfoot in previous experiments with ether-water [Miller and Chibnall, 1932].

DISCUSSION.

When the green colloidal extract referred to in the previous section was filtered through paper-pulp practically the whole of the protein passed through into the filtrate, very little being retained with the green fatty material on the paper-pad. We have always found this to be the case with the new modified ether-water method of preparation, whereas in the original experiments with ether [Chibnall and Grover, 1926] about one half of the protein was retained on the paper-pad. In former papers this fraction, which could not be readily separated from the fatty material, was referred to for convenience as the "combined" protein, while that which passed freely through the filter was referred to as the "soluble" protein. An extended research which has been made into the fatty materials present in leaves does not suggest that any true chemical combination can exist between the fats or phosphatides and the proteins, and we now believe that the "combined" protein previously obtained with ether or

"fresh" ether-water was simply a fraction of the "soluble" protein which had undergone partial dehydration or denaturation. It appears to us therefore that we have now reached a stage in the investigation of leaf proteins when such arbitrary distinctions as "combined" and "soluble" proteins have become unnecessary, and we have accordingly discontinued their use in this paper.

In comparing the yields of protein from various grasses given in Table I with that of spinach, the most successful preparation made by the old ether method, it is to be remembered that the object of the present research was to prepare the grass proteins as readily as possible in amount sufficient for analysis. The cells of spinach leaves have very thin walls and are readily disintegrated in a meat chopper or mill to give a maximum yield of protein. Blades of grass on the contrary have thick, more fibrous, cell walls, and the great labour involved in the grinding operations of a large scale preparation precludes any attempt being made to obtain maximum disintegration of the cells. Were this possible we believe that yields of 50 % or more of the total leaf protein could be readily obtained.

The protein of the grass residues is retained in unopened cells, and there seem to us no valid grounds for assuming that it differs in any way in composition from that which we obtain from the cells which have actually been torn open. We feel justified in claiming therefore that our protein preparations are representative of the whole protein of the leaf, and we shall interpret the results of our amino-acid analyses accordingly. But as we have repeatedly emphasised in previous papers there is as yet no evidence to show whether the preparations which we obtain from leaves consist of one particular protein, or whether they are mixtures of several proteins having similar physical properties.

The properties of the grass proteins are similar to those of the numerous other leaf proteins described by Chibnall and Grover [1926]. The impurity discussed at some length by Miller and Chibnall [1932] is present in all the new preparations; as a general rule we find that proteins with a high N content are obtained from young nitrogen-treated grass having a high protein content and low total dry weight, emphasising the view already expressed that the impurity is merely an adulterant with similar solubilities to those of the proteins.

SUMMARY.

The ether-water method for preparing the proteins of leaves has been modified, and excellent yields of protein have been obtained from several pure strain grasses and certain forage crops.

The essential point is that the ether-water must have been used at least once before to cytolyse an appropriate amount of leaf material. This extraordinary difference in action between "fresh" and "used" ether water is discussed in some detail.

We should like to record our thanks to Prof. V. H. Blackman for many interesting discussions during the course of this work, to the Imperial Chemical Industries for a grant to cover the cost of this research, and to Mr G. E. Blackman of Jealott's Hill Research Station for the supply of pure-strain grasses.

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CCLVII. THE ISOLATION OF *n*-TRIACONTANOL FROM LUCERNE WAX¹.

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(Received November 1st, 1933.)

In a previous paper [Chibnall *et al.*, 1931] dealing with the metabolism of paraffins in the plant the need for gaining some insight into the constitution of the mixed primary alcohols which invariably accompany them in plant waxes was emphasised. We have investigated many such mixed alcohols during the last five years, but definite evidence of their constitution was at first difficult to obtain because their components—higher primary alcohols containing 26 to 36 carbon atoms—had not then been synthesised, so that accurate standards for comparison, based on melting-points and crystal spacings, not only of these alcohols themselves, but also of the corresponding acetates, *n*-fatty acids and ethyl esters, were not available. Much of our work on plant waxes has therefore been deliberately held back pending the possible isolation from certain of them of the unknown primary alcohols in a state of unquestionable purity.

Since the work was in progress *n*-octacosanol, *n*-octacosanoic acid and *n*-triacontanoic acid have been synthesised by Bleyberg and Ulrich [1931] and *n*-triacontanoic acid by Mrs Robinson.

The preparation of pure *n*-hexacosanol from the wax of cocksfoot has already been described [Pollard *et al.*, 1931]. The presence of this alcohol, M.P. 79.8°, was readily recognised by the fact that on oxidation it gave an acid which melted at 87.9°, *i.e.* over 8° higher than the alcohol itself, whereas the mixed primary alcohols which usually occur in waxes give on oxidation acids melting only 2–3° higher, owing to the fact that mixtures of *n*-fatty acids exhibit melting-points depressed many degrees. It was to be expected that this large difference of about 8° between the melting-points of an alcohol and its corresponding acid would hold for the longer-chain primary alcohols for which we were seeking; accordingly we have oxidised a sample of every wax primary alcohol isolated in this laboratory with the hope of finding other cases in which the corresponding acid melted some 8° higher than the alcohol itself. In this we have been more successful than we had dared to hope, and have been able to prepare samples of *n*-octacosanol, *n*-triacontanol and *n*-tetratriacontanol whose purity can be vouched for by data obtained from the derived paraffins. From these alcohols we have been able to obtain the corresponding acetates, *n*-fatty acids and ethyl esters and also similar products containing one more carbon atom. Data concerning the melting-points and crystal spacings of all these substances, and of mixtures of known composition, are fully discussed in another paper.

¹ The melting-points recorded in this paper were obtained by the method described by Piper *et al.* [1931] and are corrected.

The present paper describes the preparation of *n*-triacontanol from the leaf wax of lucerne (*Medicago sativa*). This wax was first investigated at an early stage in our research when we were seeking for possible sources of ketones corresponding to the 15-nonacosanone isolated by Channon and Chibnall [1929]. Jacobson [1911; 1912] claimed to have obtained two ketones, myristone, $C_{27}H_{54}O$, and alfalfone, $C_{31}H_{62}O$, from lucerne (alfalfa) hay. We were unable to substantiate the findings of Jacobson, however, as our analysis showed that the wax was made up of fatty acids, a primary alcohol and a small amount of mixed paraffin. It was not until a later stage in our research that steps were taken to determine the constitution of this primary alcohol, and it is for this reason that the yield of pure *n*-triacontanol finally obtained falls short of the maximum amount present in the wax.

EXPERIMENTAL.

The lucerne was cut about one foot above ground, so that the sample (53 kg.) consisted chiefly of leaf material. The wax was prepared and saponified in a similar way to that described by Pollard *et al.* [1931] for the products from cocksfoot. The gross weight of wax was 49 g., equivalent to 15 % of the ether extract and 0.35 % of the dry weight of the lucerne; from it were obtained 10 g. of fatty acids which were not further characterised and 36 g. of unsaponifiable material. The constituents of the latter were then separated by the phthalate method of Chibnall *et al.* [1931].

Primary alcohol. 23 g. of unsaponifiable material gave 22.6 g. of insoluble sodium salt of a primary alkyl phthalate, which was thoroughly washed in the usual way with alcohol and ether. On hydrolysis with sodium ethoxide in benzene-alcohol it gave 18 g. of crude primary alcohol, m.p. 85°. 10 g. of this product were dissolved in boiling absolute alcohol, clarified with charcoal and the product which separated on cooling again treated twice successively in the same way. The white crystalline material thus obtained was recrystallised twice from one litre of boiling alcohol and then repeatedly from benzene-alcohol and acetone. The yield was 5 g.; m.p. 85.6–85.8°. On oxidation it gave an acid, m.p. 92.7–93.0°, with a crystal spacing (Series B. 164) of 71.5 Å., and on reduction *via* the iodide and treatment with light petroleum a paraffin m.p. 65.6–65.8° with a crystal spacing (Series B. 184) of 35.4 Å. This latter spacing, corresponding to the C form of *n*-triacontane, suggested that the paraffin must be fairly pure, showing that the primary alcohol was *n*-triacontanol. To effect if possible further purification the remainder of the alcohol was converted in the usual way with acetic anhydride into the acetate, which was then extracted repeatedly with cold light petroleum. The products thus obtained all melted within the limits 68.5–68.8°.

The alcohol recovered from the acetate crystallised from ethyl alcohol in rhombic plates with sharp angles and edges. 0.35 g. was recrystallised twice from 200 cc. of acetone at 37°. The melting-point was then constant at 86.3–86.5°, and the crystals gave a very good X-ray photograph (Series B. 259) showing 13 orders in the B or short form measuring 66.53 Å. A sample was reduced *via* the iodide (m.p. 63.8–64.2°) to the paraffin, which was treated in the usual way with sulphuric acid at 130°. The product thus obtained was extracted three times at room temperature with light petroleum (b.p. < 40°). As is usual with paraffins prepared from long-chain alkyl iodides in this way a very small insoluble residue remained. The extracted material was recrystallised from benzene-alcohol. The melting- and setting-points, transition temperatures and crystal spacings are given in Table I, the corresponding data for *n*-triacontane

Table I. *Paraffin obtained from lucerne alcohol.*

Series no.	Transition point on heating	M.P.	S.P.	Transition point on cooling	Crystal spacing in Å. C form
B. 226	60.2-60.6°	65.6-8°	65.4°	58.8°	35.46
Interpolated data	About 61°	65.6-8°	65.4°	58.8°	35.5

being obtained by interpolation from those of a series of synthetic paraffins examined by Piper *et al.* [1931]. From the discussions given in that paper there is no doubt that the paraffin obtained from the lucerne alcohol contains much less than 1 % impurity, so that the alcohol can be considered a pure sample of *n*-triacontanol.

Oxidation of the alcohol (1 g.) with chromium trioxide and glacial acetic acid [Pollard *et al.*, 1931] gave *n*-triacontanoic acid, which was recrystallised three times at room temperature from acetone. The yield was 0.8 g. and the M.P. 92.6-93.0°. Three recrystallisations from 450 cc. of acetone at 37° gave the pure acid, of M.P. 93.6-93.9° and S.P. 93.1°.

Since this work was completed Mrs G. M. Robinson has kindly placed at our disposal a sample of synthetic *n*-triacontanoic acid¹. This melted at 93.7-94° by the method used in this laboratory. Both acids gave excellent X-ray photographs in the B form (Series B. 238, 235) measuring 71.4 Å., with no trace of the C form, showing that they were pure. Bleyberg and Ulrich [1931] give the M.P. of their synthetic acid as 91.9-92.1°.

Paraffin fraction. After removal of the insoluble sodium salts of the primary alkyl phthalate in the usual way the residual material was taken up in boiling alcohol. On cooling 1.2 g. of impure paraffin crystallised out. As no appreciable amount of material representing sodium salts of secondary alkyl phthalates remained in the mother-liquor, the crude paraffin was next treated with hydroxylamine, but no ketoxime could be separated by the methods employed by Sahai and Chibnall [1932]. These two operations show conclusively that the lucerne wax contains neither secondary alcohols nor ketones. The crude paraffin was then treated with sulphuric acid at 130° until no further darkening of the acid occurred. The resulting product melted at 65.4-65.6° which is close to the M.P. of *n*-triacontane. Three simple extractions with cold petroleum (B.P. < 40°) separated it into fractions whose transition temperatures and melting-points (Table II) show that they are complex mixtures.

Table II. *Fractionation of the naturally occurring paraffin, M.P. 65.6°, isolated from lucerne.*

Fraction no.	Heating transition-point	M.P.	S.P.	Cooling transition-point
1	56.5-57.2°	64.9-65.1°	64.7°	55.2
2	57.7-58.3°	65.4-65.6°	65.3°	56.2
3	58.4-58.9°	66.0-66.2°	65.8°	56.8

The lucerne wax therefore consists of fatty acids, *n*-triacontanol and a mixture of paraffins whose mean molecular weight corresponds to that of *n*-triacontane. Jacobson [1911; 1912] extracted lucerne (alfalfa) hay with hot alcohol, filtered, evaporated the extract to dryness and took up the residue with ether. On concentration the ethereal solution deposited material which was recrystallised repeatedly from ethyl alcohol, chloroform and methyl alcohol.

¹ Unpublished work. The *n*-triacontanoic acid was obtained by reduction of 13-ketotriacontanoic acid, synthesised from ethyl 11-bromoundecate, ethyl acetoacetate and stearyl chloride by the method outlined in a previous paper [Robinson, 1930].

The product thus obtained, melted at 76.5–77° and represented 0.23 % of the hay. Portions of 0.5–0.8 g. were then treated on the water-bath for 12–15 hours with nitric acid (sp. gr. 1.104), which clarified the material with a loss of only 2.8 % by weight. The waxy cake thus obtained was extracted with hot acetone. The insoluble residue melted at 88.5–88.8° and was soluble in hot chloroform and carbon disulphide. It appeared to give no acetate with acetic anhydride and no acid on oxidation with chromium trioxide in sulphuric acid. It was therefore considered to be a ketone $C_{21}H_{42}O$ and given the name "alfalfone." No evidence for the presence of a keto-group was obtained, other than that on reduction with excess of sodium in alcohol, a product melting at 86.3–86.5° was formed. The material extracted by the hot acetone mentioned above separated as a white voluminous precipitate on cooling. It softened at 68° and melted over the range 74–77°. Because this also did not appear to react with acetic anhydride or chromium trioxide in sulphuric acid it was considered to be a ketone, and from its melting-point was identified as myristone $C_{27}H_{54}O$. On reduction with excess of sodium in alcohol it was stated to give an alcohol which softened at 72°, gave a clear meniscus at 80° and became transparent at 86°. As wax esters of long chain primary alcohols and *n*-acids are saponified only with great difficulty by hot mineral acid there is no doubt that Jacobson's "alfalfone" and "myristone" consisted of unsaponified wax esters of *n*-triacontanol mixed with paraffins, and that the change in melting-point on the assumed reduction with sodium was due to the saponification of these waxes by the sodium ethoxide produced during the reaction. There is no doubt also that the alcohol $C_{20}H_{42}O$ (M.P. 80°, B.P. 395°) isolated by Étard [1892] from lucerne leaves, and named by him "medicagol" was a mixture of *n*-triacontanol and paraffin. We shall deal in a later paper with the constitution of "myricyl" or "melissyl" alcohol, which has been considered by various workers to be either *n*-triacontanol or *n*-hentriacontanol.

SUMMARY.

The principal component of the wax from lucerne leaves is a long-chain primary alcohol (M.P. 86.3–86.5°) which has been identified as *n*-triacontanol by reduction to *n*-triacontane (M.P. 65.6–65.8°) and by oxidation to *n*-triacontanoic acid (M.P. 93.6–93.9°). The purity of all three products has been confirmed by X-ray analysis.

The wax also contains mixed fatty acids, the composition of which has not been determined, and a paraffin, M.P. 65.6° which has been shown to be a complex mixture. No ketone is present, and it is suggested that the "myristone" and "alfalfone" of Jacobson [1911, 1912] were unsaponified wax.

In conclusion we should like to thank Mrs G. M. Robinson for a sample of synthetic *n*-triacontanoic acid.

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CCLVIII. THE ISOLATION OF *n*-OCTACOSANOL FROM WHEAT WAX¹.

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(Received November 1st, 1933.)

IN continuation of the research dealing with the attempted isolation of the longer-chain primary alcohol constituents of waxes, an account is now given of the preparation from the blades of wheat (*Triticum vulgare*), of *n*-octacosanol.

Purification of the alcohol so that it could be used as a standard for our X-ray and m.p. data has proved extremely troublesome but was eventually effected in quite a simple way by repeated recrystallisation at 37° from acetone. An account, however, of some of our earlier abortive attempts at purification is given in some detail, not only because the methods employed have been used by other workers whose results we shall have occasion to criticise in later papers, but also because they illustrate very clearly the significance of the "heating transition temperature" in judging the purity of a paraffin and hence of the alcohol from which it has been derived. In the present case it has been possible to show quite definitely that the *n*-octacosanol when first isolated from the wax contains not more than about 1 % of impurity, consisting of at least three other alcohols.

EXPERIMENTAL.

Preparation and treatment of the wax. Young wheat at the stage just before the appearance of ears was cut about one foot above ground, and the blades air-dried in a room at 40°. The dried and powdered material (14 kg.) was extracted with ether, and from the material thus obtained (400 g.) the crude wax (62 g.) was prepared by the method of Pollard *et al.* [1931]. The yield was therefore 15.5 % of the ether extract or 0.44 % of the dried wheat. On saponification the wax gave 40 g. of unsaponifiable material, which was a hard yellow wax, 12.4 g. of crude fatty acids and some amorphous material which appeared to consist chiefly of chlorophyll degradation products.

As a preliminary experiment had shown that the unsaponifiable wax when treated by the phthalate method of Chibnall *et al.* [1931] gave only a primary alcohol m.p. 82–83° and a small amount of paraffin, secondary alcohols and ketones being absent, the main bulk of the material was treated in the following way to obviate the labour and losses which always attend the phthalate treatment. The material was dissolved in warm chloroform which was poured into a dish and left exposed to the air. On evaporation of the solvent a yellow friable powder was obtained, which was then shaken for ten minutes at room temperature with light petroleum (B.P. 40–60°). Twelve successive extractions removed

¹ The melting-points recorded in this paper were obtained by the method described by Piper *et al.* [1931] and are corrected.

all material (10 g.) readily soluble in this solvent, leaving 30 g. of insoluble crude primary alcohol.

Constitution of the primary alcohol. The crude primary alcohol was crystallised from carbon disulphide to remove the last traces of paraffin, giving 26.6 g. of white granular crystalline powder, m.p. 82–82.5°. 26 g. were acetylated by boiling gently for 16 hours with acetic anhydride (200 cc.) and fused sodium acetate (20 g.). The dark brown solution was poured into water and crushed ice and the mixture stirred for 15 minutes. The brown powder was collected, again stirred with iced water and finally dissolved in benzene-methyl alcohol, leaving a tarry residue. From the solution (charcoal) 27.5 g. of crystalline acetate were obtained, m.p. 63–64°. This material was fractionally distilled *in vacuo* from a Willstätter flask of 100 cc. capacity. The temperature of the metal-bath was about 280° and several fractions were collected at 185–195°/0.03 mm., the melting-points of which ranged from 63.5° to 64.5°. The higher-melting fractions were then collected and redistilled. This operation was repeated many times, but in each case the first two or three fractions melted below 64°. It was then found that if these lower-melting fractions were recrystallised twice from light petroleum (b.p. < 40°) the melting-point in each case rose to 64.4–64.6° suggesting that at the bath temperature necessary to distil the acetate a small amount of decomposition had occurred, with the formation of acetic acid and of Δ^1 -octacosene, which would be very soluble in cold petroleum. The distillation products were finally collected into four fractions, as shown in Table I, fraction 1 consisting of the

Table I. *Fractionation of the wheat alcohol acetate.*

Fraction no.	Acetate		Alcohol	Paraffin	Acid
	Wt. (g.)	M.P. (° C.)	M.P. (° C.)	M.P. (° C.)	M.P. (° C.)
1	4.1	64.4–64.6	82.6–82.9	61.3–61.5	90.1–90.5
2	5.7	64.4–64.6	82.6–82.9	61.3–61.5	—
3	8.5	64.3–64.6	82.6–82.9	61.3–61.5	90.0–90.5
4 (residue)	6.7	64.8–65.0	82.2–82.7	61.6–61.8	88.7–89.2

original low-melting samples which as mentioned above had been subsequently recrystallised from light petroleum. The corresponding alcohols were recovered by saponification and were recrystallised repeatedly from acetone and benzene-alcohol until the melting-points were constant.

Fractions 1 to 3 all melted at 82.6–82.9° suggesting that they were of uniform composition, whereas fraction 4, from the undistilled residue melted slightly lower, at 82.2–82.7°. The X-ray photograph of fraction 2 gave (Series B. 214) seven orders measuring 62.0 Å. and of fraction 3 (Series B. 160) 11 orders measuring 62.24 Å. with faint upper spacings measuring 75.46 Å. These results suggested that fractions 1–3 were practically pure *n*-octacosanol, and that the residue (4) contained a small amount of higher homologues.

A sample of each alcohol was then reduced *via* the iodide to the paraffin [Pollard *et al.*, 1931], which was treated with sulphuric acid at 130° until no further darkening of the acid occurred. After washing with water and crystallisation from benzene-alcohol, the resulting product was extracted with cold light petroleum (b.p. 40–60°). As is usually the case with paraffins prepared from long-chain alkyl iodides in this way a very small amount of white amorphous material remained insoluble. The light petroleum was removed by evaporation and the paraffin crystallised from benzene-alcohol.

The melting- and setting-points and transition temperatures of the four paraffins are given in Tables I and II. It will be seen that the distilled fractions (1-3) have melting- and setting-points identical with pure synthetic *n*-octacosane, but that the transition temperatures on heating are 2-3° lower.

Table II.

Fraction no.	Heating transition-point °C.	M.P. °C.	S.P. °C.	Cooling transition-point °C.	Crystal spacings in Å.			Orders measured
					Series no.	A	C	
1	54.8-55.5	61.3-61.5	61.1	53.4	—	—	—	—
2	55.0-55.5	61.3-61.5	61.1	53.4	—	—	—	—
3	54.6-55.0	61.3-61.5	61.1	53.3	B. 162	37.6	—	5
4	53.6-54.0	61.5-61.7	61.3	52.1	—	—	—	—
3a	54.6-55.0	61.3-61.5	61.1	53.2	B. 189	37.79	—	7
3b	54.5-55.0	61.3-61.5	61.1	—	B. 190	37.79	—	7
3c	54.5-55.0	61.3-61.5	61.1	—	B. 191	37.74	—	8
3d	55.0-55.5	61.3-61.5	61.1	53.3	B. 200	—	33.16	—
5	57.0-57.4	61.3-61.5	61.1	54.0	B. 261	—	33.21	15
Synthetic C ₂₈ H ₅₈	57.0-57.4	61.3-61.5	61.1	54.0	C. 114	—	33.4	8
90 % C ₂₈ H ₅₈ + 5 % C ₂₆ H ₅₄ + 5 % C ₃₀ H ₆₂	54.0-54.4	61.3-61.5	61.2	—	B. 70	38.0	—	5
98 % C ₂₈ H ₅₈ + 1 % C ₂₆ H ₅₄ + 1 % C ₃₀ H ₆₂	54.5-55.0	61.3-61.5	61.1	53.3	B. 168	—	33.25	6
99 % C ₂₈ H ₅₈ + 1 % (Equimolar C ₂₆ H ₅₄ + C ₃₀ H ₆₂ + C ₃₀ H ₆₆)	55.0-55.5	61.3-61.5	61.1	53.4	B. 212	37.9	—	8
98 % C ₂₈ H ₅₈ + 2 % above triple mixture	54.0-55.0	61.3-61.6	61.1	53.0	B. 213	37.7	—	5

To obtain if possible a purer sample of *n*-octacosanol the alcohol fraction 3 was passed through the phthalate treatment to remove any trace of secondary alcohol, ketone or naturally occurring paraffin. The M.P. of the resulting alcohol however was unchanged, but the X-ray photograph (Series B. 192) showed 9 orders in the short form, measuring 62.58 Å. with no trace of the longer form, which only appears at room temperature if impurity is present or when the temperature is kept above the transition-point. The derived paraffin had the same M.P., S.P. and transition temperatures as before. The latter was accordingly fractionated by means of cold light petroleum, but the three fractions finally collected (3a, 3b, 3c) showed no improvement in transition temperatures, and all gave crystal spacings (Series B. 189-191) in the A form, 37.8 Å. and were therefore impure. They were therefore united and distilled *in vacuo*. Data concerning the material thus obtained are given in Table II, 3d. It will be seen that there is an improvement of only 0.5° in the transition temperature, but that the crystal spacing now appears in the short, C form, indicating that some contamination had been removed.

Now this persistently low transition temperature for the paraffin and the difficulty of obtaining the crystal spacing in the short form might suggest—from the data for mixtures of C₂₆H₅₄ and C₂₈H₅₈ given in a former paper [Piper *et al.*, 1931]—that the paraffin was C₂₈H₅₈ with either 10 % of C₂₆H₅₄, or 5 % of C₃₀H₆₂, but the melting-point definitely excludes such a possibility. C₂₈H₅₈ with 1 % of both C₂₆H₅₄ and C₃₀H₆₂ gives too high a transition temperature on heating and a good X-ray picture in the short form. On the other hand 5 % of each of these latter paraffins gives a close approximation to the paraffin from the wheat alcohol. This result was extremely puzzling for during a prolonged fractionation of the derived paraffin referred to above we were unable to effect any displacement of the M.P. and S.P., each fraction, when melted alongside a sample of synthetic *n*-octacosane, giving parallel melting- and setting-points. Yet the transition temperature on heating was always about 2° lower than that of the synthetic paraffin. Had there been 5 % or even 2 % of impurity in the form of homologues present in this paraffin our experience leads us to believe that we

could have readily obtained a shift in the melting-point by fractionation with cold petroleum.

To check the transition temperature of our synthetic *n*-octacosane we compared it with a second sample prepared from highly pure *n*-tetradecyl iodide by the method of Backmann and Clark [1927]. In a former paper [Piper *et al.*, 1931] this sample was stated to have the correct m.p. and s.p. but to give an inferior X-ray picture. We have since found that the material can be readily purified by extraction at room temperature with low-boiling petroleum, which separates a small amount of insoluble impurity. This new paraffin gave an X-ray picture with 8 orders and a spacing of 33.27 Å. (Series B. 211) and had the same transition temperature on heating (57–57.4°) as the earlier sample of *n*-octacosane.

The X-ray pictures of all the samples of paraffin from the wheat alcohol were so unlike any of those of any even number paraffin containing a small amount of one or two other paraffins which we had yet made, that we decided to prepare some mixtures containing small amounts of three other paraffins, *e.g.* C₂₈H₅₈ with 1 % and with 2 % of an equimolar mixture of C₂₆H₅₄, C₃₀H₆₂ and C₃₂H₆₆. Data for the melting-points, transition temperatures and crystal spacings are given in Table II. Only 5 poor orders in the long form were given in both cases, and the picture given by the 1 % mixture approached nearest to, but was markedly inferior to, those given by the paraffin fractions from the wheat alcohol. The transition temperature on heating was not easily observed, but was definitely lower than that of the latter paraffins. Our investigation of the paraffins derived from fractions 1 to 3 of the wheat alcohol therefore suggested that they are *n*-octacosane with less than 1 % impurity made up of at least three other paraffins, and that consequently the alcohol was *n*-octacosanol with less than 1 % admixture of three other alcohols.

Much material and labour were expended in trying to obtain this alcohol chemically pure by repeated fractionation from the usual mixed solvents. The m.p. remained constant at 82.7–83°, and the heating transition temperature of the derived paraffin about 2–3° low. Furthermore the acid given on oxidation, which melted at 90.3–90.5°, gave an X-ray photograph (Series B. 236) with 11 orders in the B form measuring 66.73 Å. and 9 orders in the C form measuring 60.46 Å., whereas the data which we were slowly accumulating for the higher *n*-fatty acids suggested that a really pure acid should give only the B form.

Purification of the alcohol was finally effected quite simply by repeated recrystallisation from relatively large volumes of acetone, not at room temperature, which had been our usual procedure with this and other solvents, but at 37° in an incubator. 2 g. of the alcohol, m.p. 82.7–83°, were recrystallised three times in this way from 800 cc. of acetone. The yield was 0.8 g., and m.p. 83.2–83.4°, unchanged on further treatment. The X-ray photograph (Series B. 256) showed 30 orders in the B form measuring 62.15 Å.¹ The paraffin prepared *via* the iodide (m.p. 60.8–61.2°) had the correct transition temperature (57–57.4°) as shown in Table II (fraction 5) and gave an excellent X-ray photograph with 15 orders in the low or C form measuring 33.21 Å. It was a highly pure sample of *n*-octacosane, showing that the purification of the alcohol had been effective. Oxidation of the alcohol with chromium trioxide in glacial acetic acid [Pollard *et al.*, 1931] gave *n*-octacosanoic acid, m.p. 90.6–90.9°. Crystallisation at 37° from a large volume of acetone raised the m.p. to 90.8–91.1°, unchanged on further

¹ It is known that a pure long-chain compound of *n* carbon atoms usually gives very strong reflections in the *n*th and (*n* + 2)th orders. The X-ray technique has been modified during this work to allow measurement of these high orders, and an improved accuracy in the spacings has thus been obtained.

treatment. The crystal spacing (Series B. 262) was in the B form only, up to 30 orders measuring 66.76 \AA . The ethyl ester was purified by distillation ($155-165^{\circ}/0.07 \text{ mm.}$) and melted at $64.3-64.5^{\circ}$, Bleyberg and Ulrich [1931] give the M.P. of their synthetic *n*-octacosanoic acid as $90.3-90.5^{\circ}$ and of the ethyl ester as $64.8-65^{\circ}$. We shall deal in a later paper with the constitution of "montanyl" alcohol, which has been considered by various workers to be either *n*-octacosanol or *n*-nonacosanol.

Naturally occurring wheat paraffin. The 10 g. of material which were fairly readily removed from the unsaponifiable portion of the wax by means of light petroleum were dissolved in warm carbon disulphide. 2.2 g. of crude primary alcohol, M.P. $81-82^{\circ}$ crystallised out on cooling. The mother-liquor was taken to dryness and recrystallised from benzene-alcohol and acetone. 2.6 g. of white crystalline material were then obtained which were shown to be free from alcohols and ketones by appropriate treatment. This substance melted at $65-65.5^{\circ}$, and after treatment with sulphuric acid in the usual way 1.7 g. gave 1.4 g. of paraffin melting at $65.5-66^{\circ}$. As was pointed out in a previous paper [Pollard *et al.*, 1931] dealing with the wax fraction of ryegrass, which contained a paraffin with a similar M.P., earlier workers have considered such paraffins to be *n*-triacontane. As in the case of ryegrass the wheat paraffin was shown without difficulty by fractionation with light petroleum to be a complex mixture whose composition cannot at present be suggested (Table III).

Table III. *Fractionation of the paraffin, M.P. 66° , isolated from wheat.*

Series no.	Heating transition point °C.	M.P. °C.	Crystal spacing Å.	Number of order of reflection
B. 179	49 -50	61 -61.5	38.9	4
B. 178	51.5-52.5	62.8-63.3	39.6	6
B. 177	54 -55	64.5-65.0	40.6	6
B. 176	58 -59	65.2-65.7	41.1	6
B. 175	59 -60	67 -67.5	43.0	5

SUMMARY.

The principal component of the wax from blades of young wheat is a long-chain primary alcohol which has been identified as *n*-octacosanol (M.P. $83.2-83.4^{\circ}$) by reduction to *n*-octacosane (M.P. $61.3-61.5^{\circ}$) and by oxidation to *n*-octacosanoic acid (M.P. $90.8-91.1^{\circ}$). The purity of all three products has been confirmed by X-ray analysis.

The wax also contains mixed fatty acids, the composition of which has not yet been determined, and a paraffin, M.P. 66° , which has been shown to be a complex mixture.

We should like to record our thanks to the Imperial Chemical Industries for a grant to cover the cost of this research, and their Staff at Jealott's Hill Research Station, Bracknell, for the supply of young wheat blades.

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CCLIX. THE EFFECT OF ENZYMES ON THE PATHOGENICITY OF THE ROUS AND FUJINAMI TUMOUR VIRUSES.

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(Received November 2nd, 1933.)

THE effect of various enzymes on the pathological action of viruses has been investigated frequently. Glover [1931] found that trypsin had no effect on the virulence of contagious pustular dermatitis of sheep, Woodruff and Goodpasture [1929] obtained the Bollinger bodies of fowl pox in an active state by tryptic digestion of the tissue, and Jojkin and Vinson [1931] have found the virus of tobacco mosaic resistant to emulsin, pepsin and yeast extract, while it was inactivated by trypsin, pancreatin and papain. Fresh untreated tobacco juice was not affected by any enzyme. Wollmann [1925] found both trypsin-resistant and trypsin-sensitive phages, and Arnold and Weiss [1926] found that a phage was present in commercial pancreas preparations and that one phage was resistant to 4 % trypsin. Hirano [1931] carried out experiments with vaccinia and found that takadiastase and a commercial preparation of trypsin had no effect on the activity of the virus. He found that a fresh glycerol extract of pancreas inactivated it and concluded that lipase was the enzyme responsible. A low p_H of itself inactivated vaccinia so that the action of pepsin could not be investigated. Baker and McIntosh [1927] were able to inactivate the virus of the Rous sarcoma by treatment at p_H 8.0 with a commercial trypsin preparation. At p_H 6.0 they found that the same preparation had an activating effect on the virus. Sugiura [1932] also found that pepsin and trypsin inactivated the virus of the Rous sarcoma while takadiastase, Soy bean urease and castor oil bean lipase did not. He therefore concluded that the proteolytic enzymes were responsible for the inactivation. On the other hand Fränkel [1931] obtained tumours with trypsinised Rous cells and filtrates. In those experiments in which the source of the enzyme used has been stated it has always been a commercial preparation. Since these are mixtures, the results obtained by their use cannot be used to draw conclusions on the chemical nature of the virus in question. It is only by the use of separate enzymes that their true effects on the virus can be studied. In a previous paper [1931] I reported that the virus of the Rous Sarcoma No. 1 could be purified by adsorption until the infective dose contained only a very small amount of nitrogen. Similar results were obtained by Maschmann [1931] and by Lewis [1931]. The latter considered that the Rous virus could be obtained free from protein by this method. In an attempt to decide whether this was really so and if the nitrogen that remained in the purest preparations of virus obtainable by this method was then either extraneous to the virus or else peptide-nitrogen, I have investigated the action of separate proteolytic and other enzymes on the activity of the virus. I considered that if I found that the activity of the virus was not affected by any proteolytic enzymes I could draw no conclusions from the result, since it would be possible for the

virus protein to be quite resistant to their action; if, on the other hand, the virus was inactivated by a proteolytic enzyme, then the conclusion might be drawn that a protein or peptide was a constituent of the virus itself. Since Baker and McIntosh and Sugiura found that the virus was inactivated by trypsin mixtures I first investigated the action of Merck's pancreatin upon it.

Preparation of separate enzymes.

The method of separation of the enzymes in pancreatin has been described by Willstätter and his collaborators. They are conveniently summarised in Grassmann's book [1928] and the technique described there has always been followed. The preparation and estimation of the carboxypeptidase is described by Waldschmidt-Leitz [1929]. The sample of Merck's pancreatin which has been used contains an active protease, carboxypeptidase, amylase and lipase, but no aminopeptidase or dipeptidase. Waldschmidt-Leitz and Schaffner [1926] found that these latter enzymes were destroyed by treatment with acetone and ether. It is possible that these enzymes were destroyed during the preparation of pancreatin since acetone and ether are often used in drying the pancreas.

Estimation of enzymic activity.

Protease. 1.0 cc. of enzyme and 3.0 cc. of 5 % caseinogen (B.D.H.) were incubated at p_H 8.0 for 30 minutes at 37°. The increase in amino-groups was measured by formaldehyde titration with $N/10$ NaOH using phenolphthalein as an indicator. An unincubated mixture was titrated as a control.

Carboxypeptidase. The activity of the carboxypeptidase was tested by measuring the hydrolysis of chloroacetyltyrosine. 1.0 cc. of enzyme plus 1.0 cc. (sometimes 5.0 cc.) of 0.01 M substrate were incubated at p_H 7.2 for 30 minutes at 37°. The increase in formaldehyde titration was measured with $N/10$ NaOH using bromothymol blue as an indicator.

Aminopeptidase. The activity of the aminopeptidase was tested by measuring the hydrolysis of leucylglycylglycine.

Dipeptidase. The activity of the dipeptidase was tested by measuring the hydrolysis of glycylglycine.

Amylase. 1.0 cc. or less of the enzyme was incubated with 2.0 cc. of soluble starch and the achromic point was noted using iodine as an external indicator.

Lipase. 1.0 cc. of enzyme and 5.0 cc. of olive oil emulsion at p_H 8.0 were incubated for 30 minutes at 37°, and the increase in fatty acids was estimated by titration with $N/10$ NaOH in the presence of alcohol. Phenolphthalein was used as an indicator. The control was an incubated mixture of boiled enzyme and oil.

Papain. The time of liquefaction of 5.0 cc. of 6.0 % gelatin by 1.0 cc. of enzyme was found.

The effect of enzymes on the agents of the Rous and Fujinami tumours.

The tumour filtrates were all made by filtration through paper-pulp and sand according to the method of Gye and Andrewes [1926]. In all experiments, unless it is otherwise stated, equal volumes of the enzyme solution and of the tumour filtrate were mixed and incubated at 37° for two hours, and the mixture was then injected into chickens in 1.0 cc. or 0.5 cc. doses. The controls were mixtures of boiled enzyme solution and filtrate, also incubated at 37° for two hours. No buffers were added to the solutions, but the p_H was adjusted by the addition of dilute acid or alkali. No p_H shift occurred during incubation.

Table I shows the effect of fresh extracts of pancreatin on the activity of the agents of the Rous tumour No. 1 and of the Fujinami tumour grown in a hen and transplanted into a duck, and grown in a duck and transplanted into a hen. Gye and Purdy [1931] have shown that the Fujinami virus extracted from a fowl

tumour contains some substance which is neutralised by antisera to hen embryo. This substance is not present in the virus from a duck-grown Fujinami tumour. It seemed possible that an enzyme might destroy the hen substance in such a way that the virus was still able to produce a tumour in ducks though not in hens. The results show that after treatment with pancreatin the activity of the virus is inhibited in both susceptible animals. These experiments confirm the work of Baker and McIntosh and of Sugiura on the virus of the Rous sarcoma. Experiments were done with glycerol extracts of pancreatin since the separate enzymes are obtained in glycerol solution and it is known that glycerol has a stabilising effect on the virus of the Rous sarcoma [Maschmann, 1931].

Table I. *Effect of fresh pancreatin on tumour-producing power.*

In this and all other tables the number of crosses denotes the size of the tumour at the death of the bird. Four crosses represent a tumour of maximum size and one cross in brackets a tumour the size of a pea. All birds were killed one month after injection. The usual age of the hens was between three and six months. Very young ducks were used as the tumour regresses in older birds [Gye and Purdy, 1931].

	No. of bird	Control	Pan- creatin	Tumour	Enzyme preparation
(a) Ducks					
	654	XX	Neg.	Fujinami	5 % pancreatin in saline
	671	XXX	Neg.	Fujinami	5 % pancreatin in saline
	746	XXXX	Neg.	Fujinami	5 % pancreatin in saline plus 20 % glycerol
(b) Hens					
	655	XX	Neg.	Fujinami	5 % pancreatin in saline
	659	XXX	Neg.	Fujinami	5 % pancreatin in saline
	656	XXX	X	Fujinami	5 % pancreatin in saline
	669	XXX	(X)	Rous	5 % pancreatin in saline
	765	XXXX	X	Rous	5 % pancreatin in saline plus 20 % glycerol
	763	XXXX	Neg.	Rous	5 % pancreatin in saline plus 20 % glycerol
	673	XXXX	Neg.	Fujinami	5 % pancreatin in saline plus 20 % glycerol
	672	XXXX	(X)	Fujinami	5 % pancreatin in saline plus 20 % glycerol

A glycerol extract of pancreatin was then made according to the directions of Grassmann and adsorption was carried out at p_H 4.0 with alumina B in order to remove lipase. The neutralised residue contained active protease, carboxypeptidase and amylase. Usually there was also a little lipase. It was found that the enzymes contained in this fraction of pancreatin had no effect on the tumour-producing power of the filtrates. The activity of the enzymes was tested by the methods already outlined earlier in the paper and was found to be almost as great as that of the original pancreatic extract. The effect of another proteolytic enzyme was therefore tested. Papain at p_H 5.0, 6.0 and 7.0 did not inhibit tumour formation. At p_H 6.0 the same result was obtained using papain after activation with 1/1000 HCN. A 10 % extract of B.D.H. papain was so active that it caused haemorrhages in the muscles of the hens and it was necessary to kill them. All tests were then done with 1.0 % extracts.

The action of that fraction of the original pancreatin which was adsorbed at p_H 4.0 by alumina B was next investigated. The alumina was washed with glycerol and eluted with a mixture of ammonium phosphate and ammonia in glycerol and water according to Grassmann's method for the separation of lipase. The alumina was centrifuged off and the supernatant was neutralised. Glycerol to a concentration of 60 % was added to stabilise the lipase. Table II shows that the enzyme which destroys the tumour viruses is in this fraction of the pancreatin. In order to determine whether lipase was the enzyme responsible

Table II. *Effect of second fraction of pancreatin on tumour production.*

No. of bird	Control	Enzyme	Tumour	Activity of enzyme preparation			Conc. of enzyme by volume %
				Caseinogen cc.	Chloro-acetyl-tyrosine cc.	Olive oil (+CaCl ₂) cc.	
524	XXX	(X)	Fujinami	0.8	0.55	3.9	50
525	XXXX	(X)	Fujinami	0.8	0.55	3.9	50
526	XXX	(X)	Fujinami	0.8	0.55	3.9	50
512	XXXX	X	Rous	0.85	0.3	0.5	50
514	XXXX	X	Rous	0.85	0.3	0.5	50
511	XXXX	XX	Rous	0.85	0.3	0.5	50
	XXX	X	Rous	0.85	0.3	0.5	50
573	XXXX	XX	Fujinami	None	None	0.5	50
	XXX	(X)	Fujinami	None	None	0.5	66
569	(X)	—	Fujinami	None	None	0.5	75
	XXX	XX	Fujinami	None	None	0.5	50
571	XXX	X	Fujinami	None	None	0.5	66
	XXX	—	Fujinami	None	None	0.5	75

for inactivation, the effect of calcium on the action of pancreatin was investigated. The hydrolysis of olive oil by pancreatin was increased about five times by the addition of calcium chloride, but the concentration of pancreatin required just to inactivate a given tumour filtrate was not affected by the addition of this salt. This seemed to point to some other enzyme than lipase as the inactivator of the viruses. A further test of the action of lipase was made with pancreatic juice. Pancreatic juice collected from the duct contains active lipase but very little active protease before treatment with enterokinase. A sample was obtained from a dog and its action on the tumour viruses determined in the usual way. Neither before nor after activation with enterokinase was any inhibition obtained. The concentration of protease, carboxypeptidase and lipase in the activated pancreatic juice, however, was as great as in the 5 % pancreatin extracts which regularly inactivated the viruses. It is probable that the inactivating enzyme is contained in too low a concentration in freshly collected pancreatic juice to be demonstrable using the very active pulp and sand filtrates of the tumours. The experimental results obtained seem definitely against the view that the protease, carboxypeptidase or lipase in pancreatin is responsible for the destruction of the tumour viruses.

In a further study of the inactivating enzyme it was found that it rapidly disappeared from pancreatin extracts kept at 0°. The extracts were usually inactive after 7 days in the ice-chest. The addition of enterokinase does not restore the inactivating power. The proteolytic enzymes in extracts of pancreas are known to be stable whereas Willstätter and Waldschmidt-Leitz [1923] found that pancreatic lipase is unstable in solutions. This was confirmed with the pancreatin extracts used in this work. In view of the parallelism between the loss of inhibiting power and loss of lipase activity of the extracts it would be tempting to consider lipase as the inactivating enzyme, but the fact that pancreatic juice, which contains an active lipase, fails to destroy tumour-producing power is irreconcilable with this idea. It seems that an unknown enzyme is responsible for the destruction of the tumour viruses.

From these results one cannot decide whether the nitrogen remaining in the purest obtainable preparations of the tumour viruses is a constituent of the virus or not. It is possible that virus protein is resistant to the action of protease and carboxypeptidase, or the enzyme present in pancreatin extracts which destroys them may be a proteolytic enzyme of a different type.

SUMMARY.

The inactivating effect of preparations of dried pancreas on the agents of the Rous and Fujinami tumours has been investigated. A separation of the inactivating enzyme has been attempted but its identity has not been established. It is adsorbed by alumina B at p_H 4.0 together with lipase but it does not seem to be lipase. The protease and carboxypeptidase which are present in dried pancreas preparations are also inert. The inactivating enzyme is unstable at 0° in glycerol solutions.

I wish to thank the Medical Research Council for a personal grant and Dr Gye and Mrs B. E. Holmes for help in the investigation.

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CCLX. GROWTH OF BLOW-FLY LARVAE ON BLOOD AND SERUM.

I. RESPONSE OF ASEPTIC LARVAE TO VITAMIN B.

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THE experiments of Weinland [1906] upon blow-fly larvae are frequently quoted as the classical example of the synthesis of fat from protein. His work, however, is open to criticism as the analytical methods used are no longer considered reliable and the part played by bacteria was not determined. The present investigation arose out of an attempt to repeat Weinland's experiments with larvae reared aseptically on sterile blood. This diet proved inadequate for normal growth, being deficient in accessory food factors which can be supplied by yeast. The present paper deals with the response of blow-fly larvae to these growth factors. The species used was *Lucilia sericata* Meig., the Sheep Maggot Fly. These flies sometimes lay their eggs in the wool of live sheep, the larvae producing extensive sores on the skin. According to recent work in Australia [Mackerras and Freney, 1933], when maggots develop on sheep they feed at first on a serous exudate from the skin and later on blood. The nutritional value of serum and blood is therefore important in relation to the sheep blow-fly problem. Furthermore, blood is the diet of many insects which transmit diseases of man and animals.

The literature on the vitamin requirements of insects has been reviewed by Uvarov [1928] and Imms [1931] and will not be considered in detail here. Several workers have claimed that insects require "vitamin B," on the grounds that the addition of crude yeast preparations to deficient diets improves growth. Since "vitamin B" is now known to be a complex of several substances, evidence of this type does not justify the assumption that insects require the same water-soluble vitamins as higher animals. Sweetman and Palmer [1928] appear to be the only workers who have tested vitamin B₁ concentrates with insects; they reared flour beetles on a synthetic diet and found that brewer's yeast retained part of its activity after fractionation by the method of Levene and van der Hoeven; they concluded that the beetle's vitamin requirements are plural and in part identical with vitamin B₁. With regard to the vitamin requirements of blow-fly larvae, the present author found sterile muscle to be deficient in a growth factor which is present in yeast extract [Hobson, 1932, 2]; bacterial synthesis normally supplies this factor. The active substance was found to be heat-stable and it was suggested that it was not identical with vitamin B₁ or B₂. Experiments with muscle sterilised at a high temperature showed that the larvae also require a heat-labile substance present in yeast extract.

The results of the feeding experiments with blood have confirmed the composite nature of the vitamin requirements of blow-fly larvae. For normal growth

to occur under asepsis with this diet, at least three growth factors must be added: a substance absent from aqueous or alcoholic extracts of yeast, but present in a soluble form in yeast autolysate; a heat-labile factor which can be supplied by Peters's antineuritic concentrate; heat-stable substances present in autoclaved yeast extract.

EXPERIMENTAL.

Since certain micro-organisms synthesise the growth factors under examination, all the experiments were carried out aseptically. The method employed for sterilising blow-fly eggs has already been described [Hobson, 1932, 1]. Sterile horse-blood (Allen and Hanbury) was used; for each test 0.6 cc. of blood was diluted with 0.5 cc. of the test solution and absorbed on muslin bandage in a plugged test-tube. All the solutions and apparatus used were previously sterilised and the mixtures prepared with aseptic precautions. In order to coagulate the blood and ensure sterility, the tubes were then heated for 30 minutes at about 75° on 2 successive days. Three sterilised eggs were placed in each tube and the larvae reared in a moist incubator at 23–24°. In each experiment, a series of 12 to 20 tubes was run together, all the eggs being obtained from a cluster laid by one female.

Fig. 1 illustrates the effect of a deficient diet upon larval growth; these results were obtained with larvae reared without aseptic precautions upon blood absorbed on cotton-wool. With blood supplemented with an aqueous yeast extract (curve A) or marmite (curve B), normal growth occurs. It may be noted that the relative growth rate is greatest in the first 48 hours, during which the weight increases about 100 times (0.05 to 5 mg.); the larvae attain a mean weight of 50 mg. in 4 days, after which little or no further increase occurs. On blood alone (curve C) growth is nearly normal during the first 2 days; subsequently the larvae develop slowly though they will in time reach full size. In the aseptic experiments the larvae were usually weighed on the 4th day, when the maximum difference occurs; growth curves were obtained by weighing larvae from separate tubes at intervals. Sterility was checked by inoculating nutrient media from the food residues.

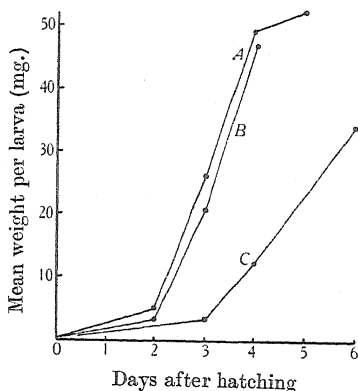


Fig. 1.

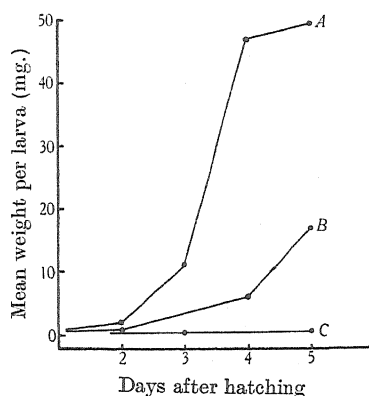


Fig. 2.

Fig. 1. Growth of unsterilised larvae on blood. A, +yeast extract; B, +marmite; C, alone.

Fig. 2. Response of aseptic larvae to marmite and yeast extract. A, blood + marmite; B, blood + yeast extract; C, blood alone. Each point was determined with a separate culture.

Response to a growth factor present in yeast autolysate.

When larvae were reared aseptically on blood alone, they completely failed to grow and weighed less than 1 mg. after 4 days. Tests were then made with blood admixed with aqueous yeast extract, fat and marmite. Yeast extract produced only a slight improvement in growth and no material difference was found when various fats including cod-liver oil were also given. On the other hand, larvae receiving marmite grew at almost the normal rate. Since non-sterile larvae develop readily upon a mixture of blood and yeast extract, it was concluded that blood is deficient in an essential growth factor which is present in marmite and is synthesised by micro-organisms; for convenience this will be referred to as the marmite factor. For studying the properties of this substance, the following basal diet was used:

Diet A. 6 cc. blood.

1 cc. concentrated aqueous extract of yeast (1 cc. \equiv 3 g.
fresh D.C.L. yeast).

0.7 cc. of this mixture was added to 0.4 cc. of the test solution.

Fig. 2 shows the growth of aseptic larvae on blood and the effect of adding yeast extract and marmite. The fact that larvae continue to grow slowly upon the basal diet (curve *B*) suggests that traces of the marmite factor are present; this is not supplied by the yeast extract since increasing the amount has no effect (Table I). Horse-blood probably contains a small amount of the active substance since larvae grow better on serum than on whole blood when an excess of yeast extract is supplied; with serum the mean weight of 4-day old larvae varied in different experiments from 20 to 30 mg. and with blood from 6 to 14 mg. If the substance is contained in the plasma, removal of the corpuscles would more than double the ratio of active substance to protein.

Table I gives the results obtained with various substances and extracts.

Table I. *The effect of various substances on the growth of aseptic larvae on a mixture of blood and aqueous yeast extract (diet A).*

Substance tested	Amount expressed as % of fresh material (or its equivalent) on volume of blood	Increase in mean weight (mg.) over negative controls after 4 days' growth
Yeast autolysate	\equiv 30	46, 40
"	\equiv 20	29, 32
	\equiv 45	44
Ditto, autoclaved 4 hours	\equiv 30	28
	\equiv 15	16
Aqueous extract of yeast	\equiv 50	2, 0, -1
70 % alcoholic extract of yeast	\equiv 50	2, 1
Aqueous extract of egg-white	\equiv 50	-1, -2
Alcoholic extract of wheat-germ	\equiv 20	0, -2
Aqueous extract of beef muscle	\equiv 40	2, 1
	25	-3
Commercial meat extract, Bovril	15	-2
	5	0
90 % alcohol-soluble fraction from peptic digest of beef	\equiv 50	37, 33
Pepsin	7	14, 12
"	3.5	6, 5
Trypsin	7	30, 33
Yeast nucleic acid	0.3	0, -2
Glycerol	1	-3, -4
Ether-soluble fraction of marmite	\equiv 30	2, 1
Olive oil	5	5, 3

Both muscle and yeast contain the marmite factor, but all attempts to obtain active extracts without digestion failed. Extracts prepared by boiling yeast with 0.1 % acetic acid, 70 % alcohol, 50 % alcohol and 50 % alcohol containing 1 % of strong hydrochloric acid proved inactive. Aqueous extracts of yeast which had been allowed to stand 3 days at about 20° appeared to be slightly active but the differences were scarcely significant; Peters's antineuritic concentrate had no effect in large doses. Active extracts were obtained by mixing yeast with half its weight of water and incubating for 8 days at 35° in the presence of toluene, the mixture being then boiled for 3 minutes and filtered. The presence of the marmite factor in muscle was inferred, since aseptic larvae grow readily on sterile muscle supplemented with aqueous yeast extract [Hobson, 1932, 2]. Successive extraction of cod-muscle with water, 50 % alcohol and 90 % alcohol failed to dissolve the substance, but an active preparation was obtained from beef muscle by the following method. Muscle was digested with pepsin solution for 10 days, the mixture filtered and the filtrate concentrated and poured into 4 volumes of 98 % alcohol. The alcohol-soluble fraction contained the active substance; control tests showed that the activity was not supplied by the pepsin.

Stability to heat. Experiments with alkalis marmite gave unsatisfactory results which could not readily be interpreted; alkaline autoclaving appears to produce substances which are either toxic or repugnant to the larvae. The marmite factor is stable to heat in weakly acid solution since extracts of autolysed yeast remained active after 4 hours' autoclaving (22 lbs.; p_H ca. 6.0).

Fractionation from marmite. An attempt was made to concentrate the active substance from marmite, the main object being to obtain preparations free from other growth factors required by the larvae. The method finally adopted was fractionation with alcohol and ether; by this means, although considerable loss of activity occurred, concentrates were obtained rich in the marmite factor but deficient in heat-stable substances essential for larval growth. The procedure varied slightly in different experiments, but the general method was as follows. An alcoholic extract was prepared by stirring 100 g. of marmite into a paste with 20 cc. of water and adding sufficient 98 % alcohol to make the concentration of alcohol 70 %, the precipitate being filtered and washed with a small volume of 70 % alcohol. The filtrate was evaporated *in vacuo* to dryness and extracted with 200 cc. of hot 88 % alcohol. This extract, which contained most of the original activity, was treated with half its volume of ether and the precipitate filtered off; the filtrate proved inactive. The ether precipitate was then extracted successively with 50 cc. of hot 98 and 90 % alcohol; both these extracts and the residue were active.

Table II shows the results of some of the feeding experiments. With crude marmite, normal growth (a mean weight of 40 mg. after 4 days) occurred with 10 % in the diet, but not with 7.5 % (percentage based on the volume of blood). 90 % alcoholic extracts, prepared by pouring 50 % marmite solution into 10 volumes of 98 % alcohol, appeared to be slightly more effective than the original product; normal growth occurred with an amount corresponding to 7.5 % of marmite. The most concentrated preparation was that obtained by extraction of the ether precipitate with 90 % alcohol, the amount of organic matter in the minimum active dose being approximately 0.3 % of the volume of blood, or 1.5 % of the dry weight; this fraction contained about 30 % of the original activity.

Attempts were also made to concentrate the active substance by precipitation and by adsorption on norite charcoal. After precipitation with normal

Table II. *The effect of marmite fractions on the growth of aseptic larvae on diet A.*

Substance	Marmite equivalent as % on blood volume	Mean weight of 4-day old larvae mg.		
		Series 1	2	3
Controls	0	7	10	8
Marmite	15	41	47	44
	10	40	43.5	—
	7.5	34	36	—
	5	19	26.5	—
Crude 90 % alcoholic extract	15	—	50	45
	10	—	53	47
	7.5	—	42	40
	5	—	35	27
90-98 % alcohol- soluble fraction	40	—	45	42
	30	—	43	45
	20	—	36	30
	10	—	17	* 15

lead acetate, both precipitate and filtrate were active but considerable loss of activity occurred. Adsorption with norite charcoal removed the active substance from extracts prepared by fractionation with alcohol, but the activity could not be recovered in appreciable amount by extracting the charcoal with *N*/10 hydrochloric acid or acid alcohol of different concentrations.

Nature of the marmite factor. The active substance in marmite is readily soluble in water, sparingly soluble in 90 % alcohol and insoluble in ether. It is not therefore a fat-soluble growth factor. Since active extracts could not be prepared from muscle and yeast without digestion, the substance is either a product of digestion, such as an amino-acid, or a soluble compound which is adsorbed by proteins or other substances that are broken down during digestion. It seems unlikely that the substance is an amino-acid since the proteins of blood and muscle do not appear to differ appreciably in their content of essential amino-acids; also, on a diet almost devoid of fat and carbohydrate, only a small part of the amino-acids would be utilised for protein synthesis and protein quality is accordingly less important. The insolubility of the substance in yeast differentiates it from most members of the vitamin B complex. However, Hunt [1928] obtained evidence that an insoluble substance in yeast stimulates the growth of rats. Williams and Lewis [1930] concluded that for optimum growth rats require a substance which is present in yeast residues after extraction and may be identical with Hunt's factor. According to Williams and Lewis, the active substance is heat-stable and insoluble in water and alcohol; in these respects it resembles the "insoluble" growth factor required by blow-fly larvae.

Response to Peters's antineuritic concentrate.

Tests with autoclaved yeast have shown that blow-fly larvae require a heat-labile growth factor which is probably identical with vitamin B₁. It may be noted that Hoagland [1923] found ox-blood deficient in vitamin B₁. Aseptic larvae were found to grow readily on horse-blood admixed with an aqueous extract of autolysed yeast, but with an autoclaved extract no growth occurred after the second day when the larvae had reached only 2 % of the maximum weight; normal growth was restored by supplementing this diet with aqueous yeast extract, alcoholic extract of wheat germ or Peters's antineuritic concentrate.

The basal diet used was prepared as follows:

Diet B. 10 cc. horse-blood.

5 cc. autoclaved yeast autolysate (1 cc. \equiv 0.9 g. fresh D.C.L. yeast).

0.9 cc. of this mixture was diluted with 0.2 cc. of the test solution. The autolysate was prepared as described on p. 1902 and autoclaved 4 hours at 22 lbs. pressure. Fig. 3 shows the growth of aseptic larvae on the basal diet and the effect of adding antineuritic concentrate; the growth rate of larvae receiving antineuritic concentrate was normal and approximately the same as that of larvae reared on meat without aseptic precautions.

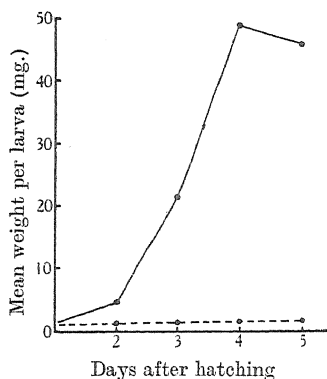


Fig. 3.

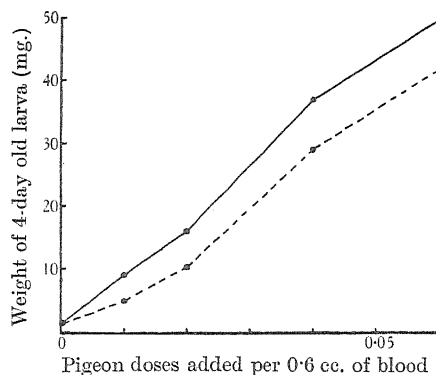


Fig. 4.

Fig. 3. Response of aseptic larvae to Peters's antineuritic concentrate. Full line, larvae receiving concentrate; broken line, controls. Each point was determined with a separate culture.

Fig. 4. Relationship between growth-promoting effect and antineuritic potency of vitamin B₁ concentrates. Full line, preparation A, pigeon dose activity *ca.* 3 mg.; broken line, preparation B, pigeon dose activity *ca.* 2.5γ.

Owing to the kindness of Prof. Peters in supplying tested concentrates of different purity, it was possible to apply a critical test as to the identity of the heat-labile larval growth factor and vitamin B₁. Two specimens of antineuritic concentrate were compared for their ability to promote larval growth; preparation A was an acid-alcohol extract having a pigeon dose activity of approximately 3 mg.; B was a purer preparation whose activity was approximately 2.5γ. The results, given in Table III and Fig. 4, show that the growth-promoting

Table III. *The response of aseptic larvae to Peters's antineuritic concentrate (basal diet B).*

Number of pigeon doses added to 0.6 cc. blood	Mean weight of 4-day old larvae				
	Series 1	2		3	
Type of preparation	A	A	B	B	
0.08	46	—	—	45	
0.06	44	50	42	38	
0.05	—	—	—	40	
0.04	39	37	29	24	
0.02	27	16	10	—	
0.01	5	9	5	—	
0	1	1.5		1	

effect of these concentrates closely followed their antineuritic activity. The amounts required to produce 4-day old larvae weighing 40 mg., when added to 0.6 cc. of blood, were approximately 0.04 to 0.05 pigeon doses of the relatively crude preparation A and 0.05 to 0.06 pigeon doses of the purer material. This agreement is surprisingly good in view of the wide difference in the nature of the biological tests involved and in the purity of the material, preparation B being about 1000 times more concentrated than A.

Kinnersley *et al.* [1933], discussing the purity of their crystalline preparations of vitamin B₁, suggest that the pigeon dose activity of the vitamin itself may be less than 1.0γ. The qualitative evidence that blow-fly larvae respond to a preparation of 2.5γ activity does not therefore prove that the effective substance is vitamin B₁ and not an associated impurity having similar chemical properties. However, the quantitative relationship observed with concentrates of different purity appears to eliminate the latter possibility, since it is unlikely that an impurity would follow the vitamin quantitatively through a process of fractionation which concentrates the activity a thousandfold. It is therefore concluded that the heat-labile growth factor required by blow-fly larvae is identical with vitamin B₁.

Response to heat-stable growth factors.

In addition to the marmite factor and antineuritic concentrate, blow-fly larvae also require heat-stable substances present in aqueous yeast extract but deficient in blood. For studying these growth factors, larvae were reared on blood mixed with antineuritic concentrate and a marmite extract prepared by fractionation with alcohol, the amounts of each being in excess of that required for optimum growth. The method of preparing the marmite concentrate was briefly as follows. An alcoholic extract was made by precipitating a suspension of marmite in water with 10 volumes of 98 % alcohol; this was treated with half its volume of ether and the precipitate extracted successively with 98 and 90 % alcohol. Tests on these extracts when mixed with blood and antineuritic concentrate showed that each produced equally poor growth; the larvae developed normally if autoclaved yeast extract was added to the diet. The basal diet adopted was prepared as follows:

Diet C. 6 cc. blood.

0.5 cc. 90-98 % alcohol-soluble fraction of marmite (1 cc. ≡ 5 g. marmite).

0.5 cc. Peters's antineuritic concentrate (1 cc. ≡ 2 pigeon doses).

0.7 cc. of this mixture was diluted with 0.4 cc. of the test solution. Since larvae grew readily on this diet when infected with a bacillus isolated from blown meat, it was assumed that the food was not deficient in mineral salts. However, later experiments showed that yeast ash stimulates the growth of larvae in the presence of bacteria, the effective ion being phosphate. Further aseptic tests were therefore carried out to determine whether the results obtained with yeast extract were due to phosphate. The following extracts were used in these experiments.

Autoclaved yeast extract. Yeast was extracted by boiling with water and the extract autoclaved 4 hours at 22 lbs. pressure.

Alkalised yeast extract. An aqueous extract was treated with a strong solution of NaOH until the p_H was approximately 10.6 and autoclaved 4 hours at 22 lbs. pressure; the solution was then made faintly acid.

Egg-white extract. An aqueous extract of egg-white was prepared by the method described by Chick, Copping and Roscoe [1930]; the sulphuric acid used to acidify the egg-white was removed by adding barium chloride solution until only traces of sulphate remained in solution.

Tests were made with these preparations to determine whether the deficient substance was vitamin B₂ or an extremely heat-stable substance which stimulates the growth of *Lucilia* larvae on sterile muscle [Hobson, 1932, 2]. Egg-white is rich in vitamin B₂; according to Chick and Copping [1930] this vitamin is destroyed in aqueous yeast extract by 4 hours' autoclaving at p_H 10.6. The larval growth factor deficient in muscle resisted 3 hours' autoclaving at p_H 9.0 in yeast extract.

Table IV. *Growth of aseptic larvae on diet C.*

Amounts refer to the equivalent fresh weight of material extracted.

Substance added	Series	Mean weight of 4-day old larvae (mg.)				
		1	2	3	4	5
Controls		12.5	11.5, 13	8, 10.5	10, 13	12.5, 13.5
Autoclaved yeast extract	10 %	39.5	40, 42.5	39, 44	—	—
Alkalised yeast extract	15 %	—	24.5, 26.5	27, 31	31, 32.5	27, 29.5
Do.	30 %	27	21.5, 23	—	—	—
Alkalised yeast extract + egg-white extract	15 %	34	31, 34	28, 30	—	—
Egg-white extract	25 %					
Do.	50 %	10	11.5, 14.5	10, 13	—	—
Sodium phosphate	0.5 %	—	11.5, 12.5	—	—	—
Infected with a bacillus isolated from larvae		—	43, 47	—	14, 15	10, 12.5

Preliminary tests with the egg-white and alkalised yeast extracts showed that both were toxic in large amount; either extract when given alone depressed growth, but the addition of both produced a slight improvement over the negative controls. The toxic effect of the alkalised yeast extract was largely removed by concentrating and pouring into 9 volumes of 98 % alcohol; the precipitate proved more effective and was used in all the experiments described. Table IV shows that this extract improved growth but could not entirely replace autoclaved yeast extract; doubling the amount of alkalised yeast extract had no further effect. The negative results obtained with sodium phosphate show that the active substance is not phosphate. Egg-white extract given alone had no effect, but appeared to improve the growth of larvae receiving alkalised

Table V. *Growth of aseptic larvae on diet C, supplemented with alkalised yeast extract.*

1.3 cc. serum were taken as equivalent to 0.6 cc. blood.

Substance added	Series	Mean weight of 4-day old larvae (mg.)			
		Blood		Serum	
		1	2	3	4
Controls		30, 33	23, 24	1.3, 1.7	1.5, 1.5
Unsterilised eggs		—	—	37, 39	33, 42
Egg-white extract	15 %	29, 35	31, 32	—	—
	30 %	27, 28	—	—	—
	45 %	39, 44	32, 37	1.5, 2.5	1.5, 2.0
	75 %	—	26, 29	—	—
Autoclaved yeast extract	10 %	40, 42	38, 39.5	44, 48	42, 43

yeast extract (Tables IV and V); however, the differences observed were small and could not always be repeated. This may have been due to the presence of toxic or repugnant substances, since the addition of egg-white extract to an adequate diet depressed the growth rate; also the basal diet contained sufficient of the factors concerned to produce fair growth.

Experiments were also carried out with horse-serum; 1.3 cc. of serum were supplied in each tube in place of 0.6 cc. of whole blood. When larvae were reared aseptically on diet C prepared with serum, very slow growth resulted; the addition of autoclaved yeast extract produced normal development, whereas alkalised yeast and egg-white extract, given either alone or together, had no effect. Table V shows the results obtained with serum, the basal diet used being diet C supplemented with alkalised yeast extract. Since larvae developed readily on this diet in the presence of bacteria, the slow growth found under asepsis is due to lack of an organic substance; this factor appears to be present in blood corpuscles since aseptic larvae grow well on this diet when it is prepared with whole blood.

These experiments show that blow-fly larvae require more than one heat-stable growth factor. The results obtained with blood (Table IV) suggest that the effect of autoclaved yeast extract is due to two substances, one extremely stable to heat and alkali, the other sensitive to alkali under the conditions used. The first substance is probably the heat-stable factor which stimulates the growth of aseptic larvae on muscle and which resembles in some respects factor "Y" [Hobson, 1932, 2]. The second substance, which is stable to autoclaving in acid medium, appears to be present in blood, but the amount is not sufficient for optimum growth; since there was some evidence that egg-white extract supplies this factor, it may be vitamin B₂. Kollath [1930] showed the presence of this vitamin in samples of alkaline haematin prepared from horse-blood. Kollath's results suggested that serum might be poorer in vitamin B₂ than whole blood, but aseptic larvae, although they failed to grow, did not respond to egg-white extract when reared on a basal diet prepared with serum. However, the marked effect of substituting serum for blood in this diet (Table V) shows clearly that the larvae require a heat-stable factor which is present in the corpuscles and is not supplied by the alkalised yeast extract.

DISCUSSION.

The results obtained with sterile blood show that blow-fly larvae resemble higher animals in their "vitamin B" requirements. Thus, the effect of yeast is due to several substances, and normal growth only occurs if each is present in adequate amount. Also, the experiments with Peters's antineuritic concentrate indicate that one of the larval growth factors is identical with vitamin B₁. The other growth factors are probably analogous to members of the vitamin B complex and they may also be identical with vitamins required by higher animals. The chief effect of "vitamin B" deficiency in blow-fly larvae was retarded growth; no evidence was obtained of loss of weight, nor were there any obvious pathological symptoms except that the larvae tended to wander away from the food. "Vitamin B" appears to act as a catalyst of growth, accelerating a reaction which proceeds in its absence at an infinitely slow rate.

It is of interest to note that Wigglesworth [1929] and also Aschner [1931] have suggested on theoretical grounds that blood is deficient in accessory food factors required by insects; this hypothesis was advanced to explain the rôle

of the symbiotic microorganisms present in insects which feed solely on blood throughout their whole life cycle. In most of these insects the alimentary tract is sterile, the symbionts being located in special organs. Aschner and Ries [1933] have recently shown that the symbionts in lice are essential for their host; extirpation of the "stomach-plates" (Magenscheibe), which harbour the symbionts, produced sexual sterility and shorter life in adult females, these effects not occurring if the operation was delayed until the symbionts had escaped from the plates. The present experiments have shown that blow-fly larvae fail to develop on blood unless "vitamin B" is added or living bacteria are present; they therefore help to confirm the hypothesis that the function of the symbionts in blood-sucking insects is vitamin synthesis.

The effect of yeast on growth is less pronounced when blow-fly larvae are reared on blood without aseptic precautions, and under certain conditions growth is approximately normal; further experiments are being carried out upon the effect of bacteria on larval growth.

SUMMARY.

1. Blow-fly larvae are unable to develop aseptically on sterile blood owing to lack of growth factors of the vitamin B type; the presence of bacteria improves growth.
2. Aseptic larvae grow at the normal rate on blood supplemented with yeast autolysate. The effect of yeast is due to an "insoluble" substance and to soluble factors which can be further differentiated by their stability to heat.
3. The "insoluble" growth factor is present in muscle and yeast and is not extracted by water or alcohol; in autolysed yeast, it is soluble in water, sparingly soluble in alcohol and insoluble in ether.
4. Peters's antineuritic concentrate supplies an essential heat-labile factor; since concentrates of different purity showed a correlation between growth-promoting effect and antineuritic potency, it is concluded that the active substance is vitamin B₁.
5. Blow-fly larvae also require at least two heat-stable growth factors, which are present in autoclaved yeast extract.
6. These results support the hypothesis that vitamin synthesis may be the function of the symbiotic micro-organisms present in blood-sucking insects.

I wish to record my thanks to Prof. P. A. Buxton for extending to me the facilities of the Entomological Department. My appreciations are also due to Prof. R. A. Peters for supplying tested samples of antineuritic concentrate and to Dr V. B. Wigglesworth for his active interest and helpful advice. I am indebted to the Agricultural Research Council for a grant which entirely financed this work.

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CCLXI. STUDIES IN AVIAN CARBOHYDRATE METABOLISM.

FURTHER STUDIES UPON THE ACTION OF CATATORULIN IN BRAIN.

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(Received October 24th, 1933.)

THIS work originated in an attempt to obtain a stable dry enzyme preparation, which would interact specifically with vitamin B₁ crystals [Kinnorsley, O'Brien and Peters, 1933]. Though this object has not been attained, the research has brought to light facts of fundamental interest not only for the vitamin function, but for the respiration of the normal brain itself [Peters and Sinclair, 1933]. It has made clear the necessity of pyrophosphate as such in the respiratory energy system of brain, which could not be so clearly distinguished without the avitaminous animal.

Former researches can be summarised [Gavrilescu *et al.*, 1932; Meiklejohn *et al.*, 1932] by saying that the typical effect of the vitamin is to increase oxygen uptake over a given period *in vitro* specifically with the avitaminous brain²; this is shown in glucose and best in lactate which is known to be formed from glucose. Respiration with succinate behaves normally in the avitaminous brain. This seems to relate the action specifically to lactate. The simple view however that catatorulin³ is the co-enzyme for lactate oxidation is not now tenable for two reasons. Boyland [1933] has found no co-enzyme effect in purified vitamin B₁ concentrates, prepared in this laboratory⁴. More significant still, Meiklejohn [1933] could obtain no evidence of an increased disappearance of lactate corresponding with the extra oxygen uptake. Hence a new hypothesis arose, namely that the effect of catatorulin was related to some other substrate, which may be called substrate X, for the full action of which upon the respiratory system the presence of lactate was indispensable. We soon found that washing the tissue reduced the oxygen uptake and lowered the vitamin effect.

It is well to emphasise that the lesion cannot lie in the cytochrome respiratory enzyme system. Lactate respiration is of course reduced by cyanide, but the catatorulin effect is completely removed. About 70 % of the O₂ uptake is sensitive to *M*/60 and *M*/600 KCN in confirmation of Dixon and Elliott [1929]. Exps. 1, Table I, and 2, Fig. 1, show this, and that *M*/600 KCN abolishes the catatorulin action. Hence this lies definitely in the part of the respiration which is cyanide-sensitive.

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² No other tissue is yet known which shows this effect.

³ Catatorulin is the substance in vitamin B₁ crystals responsible for increased oxygen uptake.

⁴ This is fully confirmed by earlier unpublished experiments by Peters.

Table I. *Cyanide and catatorulin action.*

Exp. 1.	Period	...	1st hour	2nd hour	
	L* only		1130	680	
	L + V		1330	950	
	L + KCN (<i>M</i> /60)		400	220	Av. of triplicates
	L + V + KCN (<i>M</i> /60)		380	200	

* In all Tables L=lactate; V= vitamin B₁ concentrate.

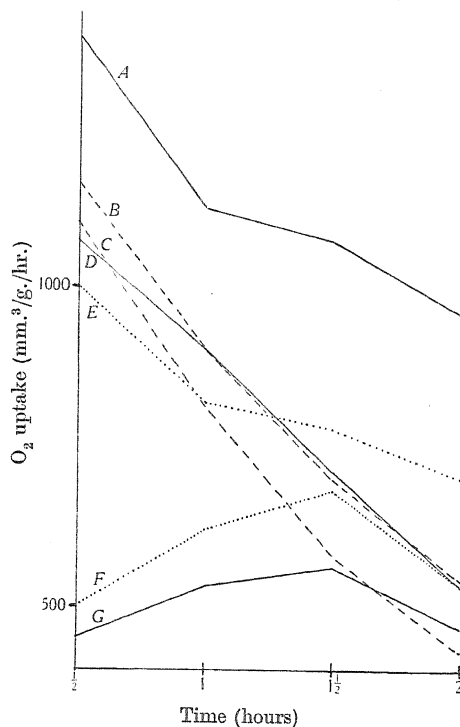


Fig. 1. (Exp. 2.) Effect of KCN and of different p_{H} upon the catatorulin effect in mixed avitaminous brain. Curves A, E, F and G p_{H} 7.3; B, C and D p_{H} 6.8. Curve A, lactate (0.033 *M*) + vitamin B₁ (1 γ /3 cc.); B, lactate + B₁ (0.2 γ /3 cc.); C, lactate; D, lactate + B₁ (1 γ /3 cc.); E, lactate (calculated from A); F, lactate + KCN (*M*/600); G, lactate + B₁ (1 γ /3 cc.) + KCN.

Since the "vitamin" effect tended to disappear upon removal of diffusible substances by washing, we turned again to a more careful study of conditions essential for catatorulin action and of the factors concerned in the maintenance of respiration in the minced tissue¹. This has revealed a system of greater complexity than at first realised; but it has also led to knowledge of some substrates which will improve oxygen uptake and so bring the respiration of the minced avian brain more into line with that of sections from mammalian brain as described by others.

The increased respiration, when vitamin B₁ is added *in vitro* to the avitaminous brain in lactate, might possibly be interpreted as a decreased rate of fall in oxygen uptake. Without added lactate, Gavrilescu *et al.* [1932] showed

¹ We prefer the term "respiration," because we feel convinced that it is more correct to consider the minced brain tissue as an organised system than an enzyme mixture.

that the catatorulin effect was present, but reduced in amount; this must be due to the low residual lactate content and shows that lactate is essential for the system.

The effect of known substrates in presence of lactate.

It is clear about the postulated substrate X, with which catatorulin interacts, that lactate is also necessary, and that it is not a substrate competing with lactate. It may arise from a precursor in the tissue; in some experiments for instance there is a distinct tendency for the extra respiration due to vitamin to increase the value $(L + V) - L$. The simplest interpretation of this is that increasing amounts of the unknown substrate are formed during the survival respiration.

Pyruvate. This substrate with the normal pigeon's brain was shown by Meiklejohn *et al.* [1932] to give a larger O_2 uptake than lactate. Hence it increases rather than diminishes respiration when added to lactate with the normal brain; *e.g.* in Exp. 1 [Peters and Sinclair, 1933], pyruvate and candiolin increase the oxygen uptake to the same extent.

The avitaminous brain behaved differently. With pyruvate alone, the oxygen uptake was never as great as with lactate and sometimes not more than the residual. Pyruvate, further, often did not interact with catatorulin. With lactate and avitaminous brain increased respiration always took place (the action has been seen in at least 300 experiments in this laboratory); with pyruvate the effect was variable¹. Further, the pyruvate system in brain recovered more slowly than the lactate, so that this failure is not directly correlated with symptoms. These facts were explained by postulating some secondary factor as necessary for pyruvate respiration.

It is essential to mention here these earlier experiments with pyruvate; they must be taken into account when trying to elucidate the course of the normal oxidative synthesis of lactate. Because vitamin B_1 often fails to improve pyruvate respiration, pyruvate alone cannot be the substrate X for which we are looking.

Three classes of respiration experiments have been tried in this search. (1) Addition of known substances in presence of lactate. (2) Addition of various extracts of brain. (3) Respiratory quotients. Only (1) is considered here². (3) forms the subject of an accompanying communication [Sinclair, 1933, 2]. (2) is not yet complete. All the experiments here recorded were made with the avitaminous pigeon brain.

Phosphorus compounds.

We have shown previously [1933] that together with lactate crude candiolin (hexosediphosphate), α -glycerophosphate and pyrophosphate all have a marked influence upon the respiration of normal brain, the last especially in inducing maintenance of respiration; β -glycerophosphate proved quite inactive.

The avitaminous brain shows general similarity with some interesting differences which are important in trying to trace the course of the biochemical reactions involved in normal respiration.

Crude candiolin and hexosediphosphate (purified). The single experiment 3a, Table II, suffices to show that hexosediphosphate alone may not have much

¹ The previous experiments with pyruvate were done with cruder preparations of vitamin B_1 . It is not known whether this introduces any difference.

² A preliminary account of some of this work was communicated to the International Congress of Cytology on 22. viii. 33.

effect as a substrate. The vitamin effect was here present, but reduced practically to the level of "residual" vitamin effect. Table II, Exp. 3b (Fig. 2), shows the effect of crude candiolin and of purified hexosediphosphate prepared therefrom.

Table II. O_2 uptake in $mm.^3$ of avitaminous brain with various additions.

Avitaminous brain. Purified hexosediphosphate (HP). Vitamin differences.

Exp. 3a.

Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1 $\frac{1}{2}$ -2			
L	1200	1080	720	230	330	400
L + V	1530	1410	1120			
HP	820	760	525	270	260	295
HP + V	1090	1020	820			

Exp. 3b.

Candiolin or hexosediphosphate.

Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2	2-2 $\frac{1}{2}$	2 $\frac{1}{2}$ -3
L	1750	1590	1300	1060	885	760
L + V	2050	1855	1550	1370	1240	1155
L + Cand.	1790	1615	1390	1210	1010	875
L + V + Cand.	2045	1940	1770	1600	1470	1370
L + HP	1810	1615	1320	1150	1015	890
L + V + HP	2040	1890	1700	1530	1425	1350

Exp. 4.

Purified hexosediphosphate.

Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2	Differences
L	1235	1040	810	715	55 $mm.^3$
L + HP	1185	1065	920	770	
L + V	1305	1190	1060	965	175 $mm.^3$
L + HP + V	1355	1290	1205	1140	

For concentrations, see p. 1924. $Q_{O_2} 5 = 1000 \text{ mm.}^3/\text{g./hr.}$

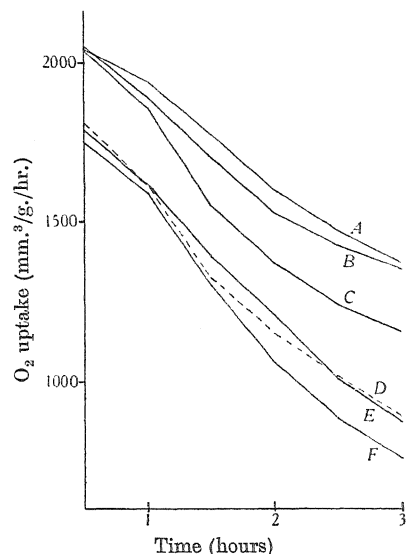


Fig. 2. Effect of candiolin and of hexosediphosphate upon the catatorulin effect in minced avitaminous brain in lactate, p_H 7.3. Curve A, candiolin (10 mg./3 cc.) + vitamin B_1 (1 γ /3 cc.); B, hexosediphosphate (8 mg./3 cc.) + vitamin B_1 ; C, vitamin B_1 ; D, hexosediphosphate; E, candiolin; F, control.

During the first hour no difference is shown; during the second hour there is a difference between preparations with candiolin and with hexosediphosphate; there is a rather greater effect in the uptake where vitamin is present in addition to these substrates. It is beyond the experimental error, but slight. In one experiment, however, out of several the effect genuinely reproduced that of the substrate sought. It is given in detail in Table II, Exp. 4.

In considering any work upon the avitaminous brain, we must keep in mind the fact that animals cannot all be in exactly the same state of avitaminosis; birds have always been selected with the greatest care. If we find animals occasionally showing a maximum effect such as this and at other times none, the interpretation is likely to be that the substrate in question in a normal animal is a remote precursor convertible into the one for which we are looking; but that the enzyme system required is temporarily more or less in abeyance in the avitaminous animal. This point is of importance later. We may note that in Exp. 4, hexosediphosphate increases the respiration mainly in presence of the vitamin.

*Robison's hexosemonophosphate*¹. In two experiments (Table III, Exps. 5, 6) Robison's hexosemonophosphate had no sustaining action, in collaboration with the vitamin. The initial effect seemed reminiscent of the normal; there was a

Table III. *Respiration of avitaminous brain with phosphorus compounds.*
O₂ uptake in mm.³

Robison's hexosemonophosphate (RHP).						
Exp. 5.						
Period (hrs.)...	$\frac{1}{2}$ -1	$1\frac{1}{2}$ -2	2-2 $\frac{1}{2}$			
L	1155	770	695			
L+V	1280	1030	920			
L+RHP	1165	770	610			
L+RHP+V	1270	1000	870			
Exp. 6.						
Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	$1\frac{1}{2}$ -2		
L	1630	1460	1150	920		
L+V	1730	1585	1360	1165		
L+RHP	1755	1490	1160	950		
L+RHP+V	1930	1680	1370	1160		
α - and β -glycerophosphate. (α -gp. and β -gp.)						
Exp. 7.						
Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	$1\frac{1}{2}$ -2	2-2 $\frac{1}{2}$	2 $\frac{1}{2}$ -3
L	1630	1460	1150	920	800	655
L+V	1730	1585	1360	1165	980	890
L+ α -gp.	1970	1815	1490	1260	1110	890
L+ α -gp.+V	2090	1975	1800	1620	1450	1310
L+ β -gp.	1430	1325	1145	910	—	—
L+ β -gp.+V	1890	1720	1410	1200	—	—
Exp. 8.						
Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	$1\frac{1}{2}$ -2	2-2 $\frac{1}{2}$	2 $\frac{1}{2}$ -3
L	1310	1190	895	725	680	560
L+V	1530	1425	1245	1150	1045	955
L+ α -gp.	1630	1520	1270	1090	930	800
L+ α -gp.+V	1685	1630	1490	1410	1335	1238
L+ β -gp.	1390	1275	1025	875	755	615
L+ β -gp.+V	1570	1460	1300	1210	1120	1030

¹ We are indebted to Prof. R. Robison for a gift of this material.

slight early stimulation of respiration. The substrate was not studied further, as it showed no promise of interest.

β -Glycerophosphate. No appreciable effect was found in experiments with this substrate which were well controlled by simultaneous experiments with α -glycerophosphate (Table III, Exps. 7, 8).

α -Glycerophosphate. The action of this substrate is in marked contrast to the others considered. It produces a large effect, amounting to some 30–40 %, and even more. But it is to be noticed that the action is not directly related to the vitamin at p_H 7.3. Even at 2–2½ hours, there is no increased catatorulin effect, *i.e.* $(L+V) - L$ is approximately the same as $(L+\alpha\text{-gp.}+V) - (L+\alpha\text{-gp.})$. It is therefore not peculiarly the substrate sought, but supplies the place of one of the missing entities in the decreased respiration of the vitamin-deficient brain.

Examination of the figures (Table III, Exps. 7, 8) shows that the effect of α -glycerophosphate is to raise the general level of respiration both with and without vitamin. There is slight increase in the differences, indicating small synthesis of X or its precursor, but hardly above the experimental error. Hence with α -glycerophosphate we have a substrate which is additive with lactate, but which shows neither maintenance nor specific interaction with catatorulin.

Na pyrophosphate. This substance is peculiarly important for the vitamin¹. At p_H 7.3, it produces a practically steady respiration for 2–3 hours, and in so doing considerably enhances the vitamin action. At p_H 6.6, where there is normally no vitamin effect, addition of pyrophosphate gives a vitamin effect. Added pyrophosphate therefore behaves as an essential constituent in a respiratory system, of which lactate and vitamin B₁ are necessary components, and therefore functions in part as our substrate.

In more than 10 experiments at p_H 7.3, we have observed the effect of pyrophosphate in a greater or less degree. There is therefore no evidence of the failure in some cases (and not in others) as seen previously with hexosediphosphate. A typical experiment is illustrated in Fig. 3.

Fig. 4 gives the effect of different concentrations (diagram at 2 hours). If we use 6 mg. of the Na salt, the effect is therefore practically maximum.

The conclusion is that lactate and free pyrophosphate in sufficient concentration are essential for the action of vitamin B₁; from this follows naturally the inference that the interaction of these three substances constitutes the basis of the catatorulin effect and is essential for normal tissue respiration. This could only have been learnt by a study of the avitaminous bird.

Mixed substrates. Some experiments upon the effect of mixed substrates have given results rather similar to those in the normal; the additions do much to restore normality in the avitaminous bird.

Table IV shows the very large increases which may be expected by these additions to the avitaminous brain, pyrophosphate, vitamin and α -glycerophosphate together with lactate inducing rises of at least double and in the case of Exp. 11 nearly treble the respiration rate as compared with lactate alone.

Adenosine triphosphate. The pyrophosphate may either function as the free salt, or because it assists in the resynthesis or preservation of the co-enzyme

¹ We were led to this discovery by an attempt to synthesise phospholactic acid by heating lactic acid with metaphosphoric acid. Upon neutralising on ice with 20 % NaOH, crystals separated, a solution of which had marked maintenance activity. The isolated barium salt subsequently proved to be barium pyrophosphate.

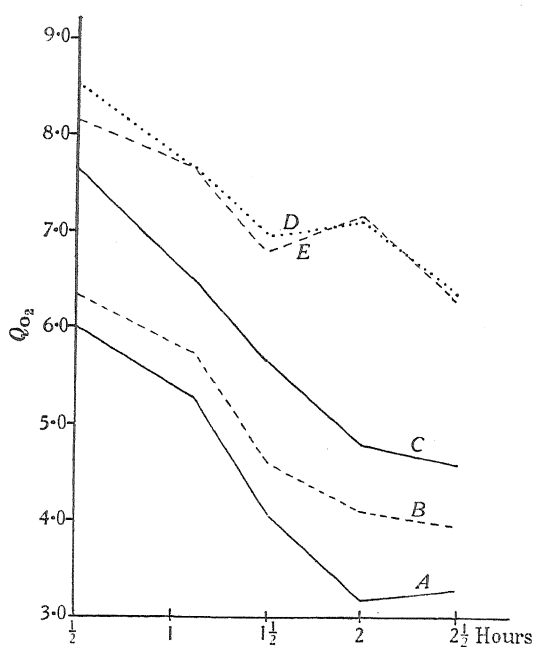


Fig. 3. Effect of pyrophosphate upon the respiration of avitaminous brain in lactate p_H 7.3. Curve A, control; B, pyrophosphate (6 mg.); C, vitamin B_1 (1γ); D vitamin and pyrophosphate (6 mg.); E, vitamin and pyrophosphate (12 mg.).

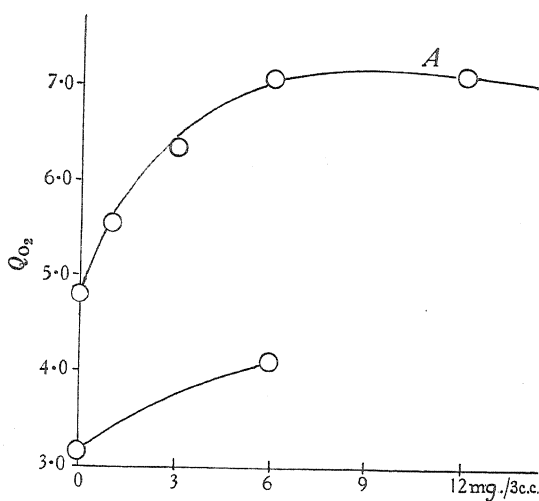


Fig. 4. Effect of concentration of pyrophosphate upon respiration of avitaminous brain in lactate, p_H 7.3. Curve A, with addition of vitamin B_1 (1γ/30 cc.).

Table IV. *Mixed substrates**. *Avitaminous brain. O₂ uptake in mm.³ P_H 7.3.*

	Period (hrs.)	...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2	2-2 $\frac{1}{2}$	2 $\frac{1}{2}$ -3
Exp. 9.	L alone		1160	970	710	730	560	500
	L+V		1200	1145	1010	880	860	760
	L+pp.+V		1070	1115	1105	980	1030	880
	Same+ α -gp.		1460	1420	1315	1230	1190	1040
Exp. 10.	L+ α -gp.		1720	1640	1280	1125	815	—
	L+pp.		1360	1050	700	730	820	—
	L+pp.+V		1640	1360	1300	1260	1290	—
	L+pp.+ α -gp.		1930	1495	1290	1140	1000	—
	L+pp.+ α -gp.+V		1840	1890	1680	1510	1460	—
Exp. 11.	L		715	600	—	—	—	—
	L+pp.		1010	880	750	715	540	—
	L+V		1030	1020	—	—	—	—
	L+pp.+V		1120	1120	1070	1000	860	—
	L+ α -gp.		1225	1100	1040	930	782	—
	L+pp.+ α -gp.		1390	1330	1210	1050	870	—
	L+pp.+ α -gp.+V		1525	1620	1495	1335	1170	—

pp. = pyrophosphate.

* It seems most probable (but lacks proof), that these are the actual missing substrates in the tissue itself, and that the initial varying rates of respiration in lactate alone are due to varying amounts of these substances in the tissue preformed perhaps during the stimulation of killing and subsequent manipulation. Some preliminary evidence has already been obtained in support of this.

adenyl pyrophosphate [Lohmann, 1928; 1932]. We have obtained no vitamin or vitamin-pyrophosphate effect by addition of the co-enzyme up to concentrations of approximately 2 mg./3.0 cc. = 0.07 %. Some earlier negative experiments with a crude preparation of our own have been confirmed with a specimen very kindly given to us by Dr Boyland¹ (Table V, Exp. 12).

Table V. *Adenosine triphosphate (Adp.) 2 mg./3 cc. Exp. 12.*
O₂ uptake in mm.³

	Period (hrs.)	...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2
L			1210	890	690	550
L+Adp.			1150	800	630	480
L+pp.			1230	910	820	650
L+V			1330	1090	920	710
L+Adp.+V			1510	1090	(925)	715
L+pp.+V			1380	1180	1090	990

Since increase in concentration of adenyl pyrophosphate has no influence in presence or absence of the vitamin, it must be concluded that free pyrophosphate is the essential component of our system, and that this is acting independently of the co-enzyme in this particular respect.

Note. Nagai [1932] has recently studied the pyrophosphate content at death of the muscles of avitaminous pigeons. He found no difference from normal for breast and heart muscle, but a 15 % decrease for avitaminous leg muscle. The latter is barely significant statistically. The figures are in any case of doubtful value, because there is no statement of time elapsing between death and immersion in cooled trichloroacetic acid.

¹ We gratefully record our thanks for a gift of Ba adenosine triphosphate. This was decomposed in acid solution (HCl) and checked by estimation of inorganic and total P and P hydrolysable in 7 minutes.

Hydron concentration and the catatorulin effect.

With normal brain [Peters and Sinclair, 1933] the O_2 uptake at p_H 6.6 is approximately 60–70 % of the value at p_H 7.3. Respiration in lactate solutions is of course lower for avitaminous than for normal brain. The difference between these two hydron concentrations is not nearly so marked for avitaminous brain in absence of added vitamin; but whereas the presence of vitamin increases respiration at p_H 7.3 markedly, this increase with added vitamin is much reduced or even absent at p_H 6.6. Table VI shows typical experiments (a) comparing lactate and lactate + vitamin at p_H 6.6, (b) lactate and lactate + vitamin at p_H 6.6 and 7.3 (see also Fig. 1, Exp. 2).

Table VI. *Effect of p_H on vitamin action. O_2 uptake in mm.³*(a) L + V (1 γ at p_H 6.6).

Period (hrs.)...	Exp. 13		Exp. 14	
	0-1	1-2	0-1	1-2
L	810	470	1000	610
L + V	900	550	1135	775

(b) L + V (1 γ) at p_H 6.6 and 7.3.

Period (hrs.)...	Exp. 15		Exp. 13	
	0-1	1-2	0-1	1-2
p_H 7.3	1265	1020	1290	1090
p_H 6.6	990	630	900	550

(c) p_H 6.6. V 0.2 γ and 1 γ .

Period (hrs.)...	Exp. 16	
	0-1	1-2
L	955	505 (triplicate averages)
L + 0.2 γ V	1030	620
L + 1 γ V	990	630

It is of considerable interest that the catatorulin effect should be reduced from a maximum at p_H 7.3 (the blood- p_H) to a minimum at p_H 6.6. A further point of importance is that any slight vitamin effects at p_H 6.6 are obtained with small amounts of vitamin (0.2 γ); but increase of vitamin to 1 γ does not give increased respiration, as at p_H 7.3. The equation of Passmore *et al.* [1933] does not hold at p_H 6.6 (see Table VI (c)). This was remarkable, and at first seemed to be explicable only by some abrupt inhibiting effect. The probable explanation has proved more interesting. If pyrophosphate is added, there is a vitamin effect at p_H 6.6, similar to that at p_H 7.3 (see Table VII). Hence, sufficient pyrophosphate must be formed at p_H 7.3 to give the ordinary vitamin effect; this pyrophosphate formation largely fails at p_H 6.6. The reason for this is still obscure; the optimum p_H for pyrophosphatase action was given by Kay [1928] as p_H 7.6, but since then pyrophosphatases with other p_H optima have become known [see Takahashi, 1932].

There is a distinct suggestion that the origin of this pyrophosphate may be normally related to α -glycerophosphate. Though α -glycerophosphate acts independently of the vitamin at p_H 7.3 so far as oxygen uptake is concerned, at p_H 6.6, α -glycerophosphate functions to some extent in place of pyrophosphate (see Table VII, Exps. 17 and 18).

The striking influence of pyrophosphate at p_H 6.6 lends the strongest support to the view that free pyrophosphate is a component of normal cell respiration in brain tissue. The concentration of adenosine triphosphate in 100 mg. tissue

Table VII. *Respiration at p_H 6.6. O_2 uptake in mm.³*

Pyrophosphate. α -Glycerophosphate. Vitamin.						
Exp. 17.	Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2	
	L	1165	915	710	520	
	L + V	(1175)	(985)	(780)	(635)	
	L + α -gp.	1440	1010	740	510	
	L + α -gp. + V	1615	1230	970	695	
	L + pp.	945	810	730	640	
	L + pp. + V	1410	1210	1060	950	
Exp. 18.	Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2	
	L	960	800	530	455	
	L + pp.	960	770	685	580	
	L + V	1150	915	710	540	
	L + V + pp.	1200	1115	1050	940	
Exp. 19.	Period (hrs.)...	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2	2-2 $\frac{1}{2}$
	L	890	735	640	390	340
	L + V	1040	835	625	490	370
	L + pp.	955	840	690	685	610
	L + pp. + V	1225	1145	1035	1010	1060
	L + α -gp.	930	835	770	640	470
	L + α -gp. + V	1120	1060	955	850	810

is low, so that such pyrophosphate can hardly arise from this source, even if the α -glycerophosphate action is otherwise explained. It must be mentioned again in final caution that these findings with substrate additions still require the experimental proof by estimation of concentration in the tissues concerned before we can be quite certain of their application.

The above interesting facts unfortunately do not yet teach us the precise nature of the enzyme system with which the vitamin co-operates. Though pyrophosphate behaves as substrate X, its action must be in some way secondary; *i.e.* the postulated substrate X is multiple. In the attempt to learn whether this was concerned with the accepted hexosephosphate fermentation system, we investigated the action of fluoride.

Fluoride.

The effect of fluoride upon tissue respiration has been much studied [see Meyerhof, 1930]. In mammalian brain, Ashford and Holmes [1929] showed that 0.02 *M* fluoride inhibited lactate formation by 88 %, 0.01 *M* by 81 %, 0.005 *M* by 72 % and 0.002 *M* by 64 %. It is uncertain however that this inhibition is due to stabilisation of hexosephosphates, as even 0.02 *M* did not inhibit the appearance of inorganic phosphate under these conditions (p_H 8.2). At first it was thought that the vitamin effect was relatively little influenced by fluoride. Further experiment showed however that this was a question of concentration.

Table VIII gives the experiments. In each case 0.024 *M* NaF reduced the respiration of L+F+V to that of L+F; this concentration reduced the lactate respiration by 30-35 %, a figure which must be approximately maximum as 0.048 *M* gave no further striking change in L+F. In the second hour period the reduction with 0.024 *M* formed a rather greater percentage of the total respiration, but in actual amount (mm.³/g./hr.) was not much different from the reduction in the first hour. About 300 mm.³/g./hr. are sensitive to fluoride under

Table VIII. *Effect of fluoride (NaF). O₂ uptake in mm.³*

Exp.	Period (hrs.) ...	0-1	1-2	Calculated differences		Total
				1st	2nd	
Exp. 20.	L	940	570	330	280	(610)
	L + F (0.048 M)	610	290			
	L + V	1160	800	380	475	(855)
	L + F (0.024 M)	780	325			
	L + α -gp.	1370	1010	280	320	(600)
	L + F (0.024 M)	1090	690			
Exp. 21.	L	1030	600	190	210	(400)
	L + F (0.008 M)	820	390	190	100	(290)
	L + F (0.024 M)	630	290	530	490	(1020)
	L + V	1200	780			
	L + F (0.024 M)	670	290			
	L + F (0.024 M) + α -gp.	590	310	—	—	
	L + F + α -gp. + V	640	390			
Exp. 22.	L	1160	690	190	200	(390)
	L + F (0.008 M)	970	490	160	60	(320)
	L + F (0.024 M)	810	430	420	440	(860)
	L + V	1480	960			
	L + F (0.008 M) + V	1060	520			
	L + F (0.024 M) + V	855	450	205	70	(278)
	RP	855	460	270	210	(480)
	RP + F	585	250			
Exp. 23.	L	1395	850	160	230	390
	L + F (0.004 M)	1230	600	90	150	240
	L + F (0.008 M)	1140	450	340	390	730
	L + V	1780	1120			
	L + F (0.004 M) + V	1440	730			
	L + F (0.008 M) + V	1150	485	260	245	500
	RP	805	450	140	60	200
	RP + F (0.004 M)	665	350			

RP = Ringer-phosphate.

our conditions. 0.008 M NaF inhibited catatorulin action completely in two experiments and only partially in a third. With 0.004 M the catatorulin effect was present in Exp. 23, but inhibited some 50 % (reckoned as mm.³/g./hr.). 0.01 M therefore appears to be the marginal concentration for complete inhibition of the catatorulin effect; this will only reduce the lactate respiration by 18 %. The catatorulin effect is therefore much more sensitive to fluoride than 70 % of the tissue respiration in the presence of lactate; it appears to follow the concentrations required to inhibit lactate formation more closely, but to be less sensitive than the lactate system.

Some other points deserve notice. From the fluoride action it may be judged that about 70 % of the residual respiration is due to lactate in the first hour, and in the second hour much more. In presence of lactate, the proportion of fluoride-sensitive respiration increases in the second hour. If fluoride inhibits the hexosephosphate-triosephosphate system, this means that more of these compounds are taking part in the respiration in the second hour; they are therefore formed from precursors in the first hour. In presence of vitamin, the

amount of such substances formed must be much greater; in Exp. 22 for instance some 600 mm.³ of the respiration are inhibited by fluoride. This suggests that the vitamin is concerned somehow in the fermentation system of carbohydrate, stimulating a respiration which is fluoride-sensitive¹. The extra respiration is intermediate in sensitivity to fluoride between lactate upon the one hand and the main tissue respiration on the other.

The concentration 0.01 *M* fluoride required to inhibit the vitamin effect is practically the same as that shown by Dickens and Greville [1932] to inhibit glucolysis in the Jensen rat sarcoma.

To recapitulate, the simplest hypothesis that vitamin B₁ is directly concerned with lactate removal was replaced by the hypothesis that it interacted with an unknown substrate X. Now it seems that X is more than one substance.

Further considerations.

The results upon normal and avitaminous brain suggest a close relation to the new fermentation scheme of Embden *et al.* [1933], in which Meyerhof and McEachern [1933] concur. The powerful action of α -glycerophosphate makes this evident.

According to these authors, we have for the anaerobic system:

- (1) Hexosephosphoric acid \rightarrow 2 triosephosphoric acid [dihydroxyacetonephosphoric acid (*a*) + glyceraldehydophosphoric acid (*b*)].
- (2) (*a*) + 2H + (*b*) + O = α -glycerophosphoric acid + phosphoglyceric acid.
- (3) Phosphoglyceric acid = pyruvic acid + phosphoric acid.
- (4) α -Glycerophosphoric acid + pyruvic acid = triosephosphoric acid + lactic acid
(= 2 lactic acid + phosphoric acid) [Meyerhof and Kiessling, 1933].

In (4) pyruvic acid formed from phosphoglyceric acid in presence of α -glycerophosphate gives lactate (salt at p_H 7.3).

The prominent part played by pyruvic acid (as opposed to methylglyoxal) in these new fermentation schemes makes necessary a further investigation of pyruvate in relation to these brains. The primary object of this paper was confined to investigation of the respiration, but a few other incidental observations have also been made.

Tested by the nitroprusside reaction of Simon and Piaux [1924] as described by Case [1932], no pyruvate reaction is given by the freshly minced avitaminous brain. After shaking with lactate-Ringer at p_H 7.3 for periods up to 2 hours, there is usually found a marked pyruvate reaction.

It is probable that the reaction is actually given by pyruvate, but this cannot be decided until a hydrazone has been separated and identified by melting-point. The reaction is peculiar to avitaminous brain at p_H 7.3. It is not found in experiments carried out at p_H 6.6, or in normal brain tissue. Hence, it is in some way related to the avitaminosis; the obvious connection with Embden's scheme is of great interest.

Upon the assumption that this substance will prove to be pyruvate itself, two problems arise for further work, namely (1) the origin and disappearance of

¹ During the progress of these experiments, Meyerhof's view [1933] that fluoride inhibits phosphoglyceric acid breakdown appeared. It is difficult to reconcile this effect of fluoride with the view that fluoride only influences the breakdown of phosphoglycerate.

the substance responsible for the pyruvate reaction in the respiration experiments, and (2) the nature of the pyruvate lesion.

In regard to (1), two paths are available upon present knowledge, either oxidation of lactic acid or degradation of phosphoglycerate. Origin by oxidation *via* the lactic co-enzyme of Banga, Szent-Györgyi and Vargha [1932] cannot be excluded, unless it proves that the lactic co-enzyme functions well at p_H 6.6; at this p_H no pyruvate reaction appears. The more probable origin appears to us to be *via* Embden's phosphoglycerate; this would account among other things for the ready disappearance of the reaction in presence of α -glycerophosphate; further, since lactic acid would be so formed, it would go far towards explaining the puzzling constancy of lactate in Meiklejohn's experiments [1933]. (2) The problem as to the nature of the pyruvate respiration lesions in these brains has still to be solved. Respiration with avitaminous brain in pyruvate solutions in absence of added vitamin is almost always less than with lactate, in contrast to the normal. The previous results with vitamin additions were variable [Meiklejohn *et al.*, 1932]. A few further experiments show that addition of pyruvate to lactate may be actually inhibitory (see Exp. 24, Fig. 5). At other

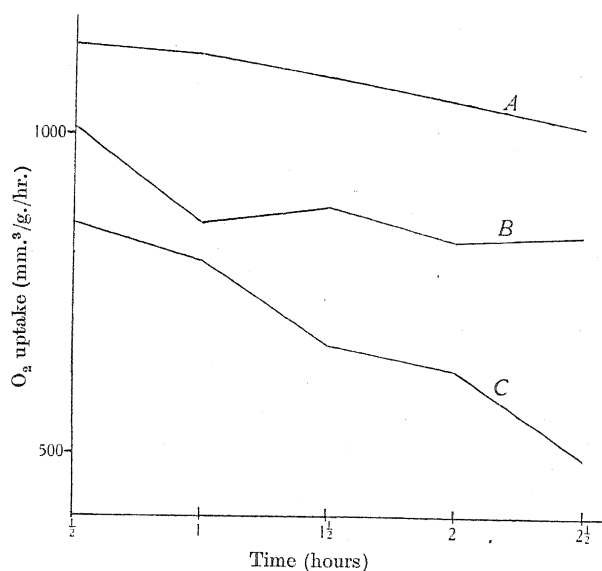


Fig. 5. (Exp. 24.) Effect of pyruvate upon catatorulin effect in minced avitaminous brain in presence of lactate (0.033 *M*) and pyrophosphate (7.5 mg./3 cc.). Curve A, vitamin B₁ (1γ/3 cc.); B, vitamin B₁ + pyruvate (2.8 mg./3 cc.); C, control.

times an experiment such as Exp. 25, Table IX is seen, giving reduced respiration with pyruvate, but identical catatorulin effects with pyruvate and lactate. At present the balance of evidence is towards the view that the pyruvate problem is secondary to that of lactate, so far as the action of the vitamin is concerned. Two paths seem possible for removal of pyruvic acid, synthesis to phosphoglycerate, or reduction to lactate. Exp. 25 is best explained by believing that pyruvate is first reduced to lactate. If then, the presence of vitamin also improves removal of pyruvate by reduction to lactate, the vitamin must be a

Table IX. O_2 uptake in mm.³

Table IX. <i>O</i> ₂ uptake in mm. ³				
Exp. 25.				
Period (hrs.) ...	1	2		V difference
Ringer-phosphate	1030	590	}	110 mm. ³
Ringer-phosphate + V	1080	700		
L	1600	1010	}	360 mm. ³
L + V	1810	1370		
Pyruvate	1360	850	}	330 mm. ³
Pyruvate + V	1580	1180		
Exp. 26.				
Period (hrs.) ...	1	2	3	Difference Total 1-3 hrs.
L	1400	750	615	690
L + V	1560	1040	860	
P	1080	680	380	1020
P + V	1490	960	730	
P + α -gp.	1460	860	690	910
P + α -gp. + V	1810	1160	950	

catalyst in some reducing system. We cannot yet decide which substance reduces pyruvate when oxygen is present. According to Barrenscheen and Beneschevsky [1932] it would be glutathione; whereas according to the scheme of Embden *et al.* [1933] and Meyerhof and McEachern [1933] it would be preformed α -glycerophosphate. If α -glycerophosphate can actually take the place of oxygen, then it might be expected that α -glycerophosphate + pyruvate would give less respiration than pyruvate. In three experiments of which Exp. 26, Table IX, is one, respiration with α -glycerophosphate addition has been increased additively with pyruvate, as with lactate. This independence of α -glycerophosphate respiration is decidedly puzzling, and at the moment difficult to reconcile with the latest fermentation schemes. There is no support at present for the view that the vitamin action is concerned with α -glycerophosphate, or directly with pyruvate, though the fluoride experiments suggest a relation to the fermentation system of carbohydrate¹. We have come to believe that the changes in the tissue induced by lack of vitamin are more profound than at first realised, and that the whole trend of metabolism is altered; this is indeed indicated by Meiklejohn's and also by the R.Q. experiments [Sinclair, 1933, 2]. Since Hopkins and Elliott [1931] showed that pyrophosphate inhibited glutathione oxidation, the interaction with pyrophosphate would be consistent with some such function for the vitamin as mobilising active glutathione in a coupled oxidation system. Knowledge of the exact influence will come only with the patient accumulation of further facts.

The deep-seated changes experienced outwardly by increased lactate in the tissue here studied and variations in normal respiration are especially interesting, because of their close analogy to those in tumour tissue, which has been made closer by work upon the R.Q. Dickens and Simer [1930] found that this was the great distinction for tumour (a lowering in carbohydrate oxidation in addition to lactic acid formation). Here analogy stops because there is no evidence that polyneuritic pigeons grow tumours in their brains more easily than the normal.

¹ An experiment with ketol [Henze, 1930] gave no indication of change in the respiration either with or without vitamin. We are indebted to Dr Boyland for the specimen of ketol.

The above research has been carried out in Ringer-phosphate solution. The criticism that conditions are too artificial to be of interest is adequately met by the recent finding (H. M. S.) that the catatorulin effect is obtained equally well in bicarbonate- CO_2 solutions.

EXPERIMENTAL.

Birds used for these experiments have been carefully selected to show true opisthotonus symptoms. They have in most cases been already used in vitamin B_1 tests and have therefore been thoroughly depleted of vitamin.

Determinations have been made upon the whole minced brain (up to 16 simultaneous samples) in Ringer-phosphate p_{H} 7.3, containing lactate 0.033 M unless otherwise stated, and in O_2 . The salts used have been described in our previous paper [1933]. The figures given represent the mean of satisfactory duplicate estimations, unless otherwise stated. Periods are calculated for 30 minutes preceding the time stated, *i.e.* $1\frac{1}{2}$ hours means the period $1-1\frac{1}{2}$ hours.

In making additions to the Ringer-lactate solution, the salts have been made up in 0.2–0.3 cc. of solution and so added to the bottles, making allowance for this by addition of less Ringer-lactate. Any changes in concentration of lactate so produced would not influence results [*cf.* k_m curves for lactate, Gavrilescu *et al.*, 1932]; but this has been controlled in the latest experiments by adding the necessary amount of lactate in 2.0 cc. Ringer-phosphate and making up to 3.0 cc. with the various additions. The small variations in tonicity have not been corrected. It was found by Peters and Gavrilescu that very wide variations in strength of a sugar solution had little influence upon oxygen uptake.

The same batch of vitamin crystals has been used throughout. They were made up as described by Kinnersley *et al.* [1933] in acid alcoholic solution, 200 γ /cc. The required amount was taken immediately before an experiment, the alcohol removed, and diluted with Ringer-lactate so that 1 cc. = 2 γ . It was added to the bottles immediately after breaking up the tissue.

Magnesium. As in the previous paper, Mg has been added as 0.05 cc. of saturated Mg phosphate solution in several cases (Exps. 3, 5, 6, 7, 8, 10, 11, 12, 17, 19). It has had no influence.

Calcium concentration. Calcium chloride added to the Ringer-phosphate caused at p_{H} 7.3 inhibitory effects amounting to some 2–10 %, but did not affect the action of the vitamin. In order to work under standard conditions, most of the experiments were done with Ringer-phosphate which was allowed to stand at room temperature for 2 days before filtering from the precipitated calcium phosphate. Latterly, we have worked with a Ca-free Ringer without influence upon the experimental results. For other work upon the influence of calcium see Kisch [1931] and for nerve Gerard [1932].

Concentrations of other substrates. Unless otherwise stated, these have been as follows:

Vitamin B_1 . 1 γ per 3.0 cc. ($\frac{1}{2}$ International vitamin B_1 unit).

Pyruvate 0.019 M . Exps. 26, 27.

Pyrophosphate (Na salt). Anhydrous: 0.0094 M , Exps. 12, 17, 18, 19: 0.0075 M , Exps. 10, 11: 0.0125 M , Exp. 9.

α -Glycerophosphate (Ca salt decomposed by oxalate): 0.0143 M , Exps. 7, 8: 0.0238 M , Exps. 9, 11, 17, 19: 0.02 M , Exp. 27: 0.016 M , Exp. 10.

β -Glycerophosphate (Na salt): 0.0129 M , Exps. 7, 8.

Hexosemonophosphate (Robison) Ba salt: 0.0076 M , Exps. 5, 6.

Hexosediphosphate Ba salt: 0.0071 *M*, Exps. 3*a* and *b*, 4.

Candiolin. 10 mg./3 cc., Exp. 3*b*.

They were chosen to produce maximum (or approximately maximum) effects.

CONCLUSIONS.

1. The action of vitamin B₁ "catatorulin effect" upon the brain *in vitro* of avitaminous pigeons is maximum at p_H 7.3. It is abolished by cyanide and fluoride in sufficient concentration, being more sensitive to fluoride than most of the respiration.

2. Na pyrophosphate (0.2 %, 0.01 *M*) interacts with vitamin B₁ and lactate to produce large rises in respiration over periods of 2–3 hours; in this way the respiration is largely maintained instead of decreasing rapidly.

3. Hexosediphosphate and Robison's hexosemonophosphate increase the initial rate of respiration in avitaminous as in normal brains, but have no influence in sustaining respiration and show no specific vitamin interaction.

4. α -Glycerophosphate (not β -glycerophosphate) increases the respiration largely; this increase is not specifically related to the vitamin action, but the substance is probably one of the missing tissue substrates in experiments of this type.

5. With lactate alone at p_H 6.6 there is no catatorulin effect unless pyrophosphate is added. To some extent α -glycerophosphate will replace pyrophosphate. It is inferred from this that free pyrophosphate is split off in survival respiration at p_H 7.3.

6. Since vitamin B₁, lactate and pyrophosphate appear to form some coupled oxidation system, the vitamin must influence more than one phase of the energy metabolism of the cell. The facts at present known are the expression of some deep-seated shift in cell equilibria.

We are indebted to the Medical Research Council and to the Christopher Welch Trustees for grants for expenses. We are grateful to Mr H. W. Kinnersley for help in preparation of the vitamin concentrates and to R. W. Wakelin for skilful assistance.

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CCLXII. THE EFFECT OF VITAMIN B₁ UPON THE RESPIRATORY QUOTIENT OF BRAIN TISSUE.

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(Received October 31st, 1933.)

GAVRILESCU AND PETERS have shown [1931, 1] that minced polyneuritic pigeon's brain tissue, suspended in glucose-phosphate solution, gives a lowered oxygen uptake as compared with the normal. Later they found [1931, 2] that this deficiency was partially restored *in vitro* by adding vitamin B₁ concentrates in very small amount. The effect was catalytic. Peters and his colleagues [Gavrilescu *et al.*, 1932] showed that the effect was enhanced in lactate solution; but since the avitaminous tissue oxidised succinate normally, the cytochrome system was not impaired. They concluded that vitamin B₁ was specifically concerned with carbohydrate metabolism.

The simplest explanation was that vitamin B₁ was a catalyst essential for the oxidative removal of lactic acid. It is not, however, the lactic oxidase coenzyme described by Banga *et al.* [1932], neither can preparations of the latter give the *in vitro* effect of vitamin B₁ [Boyland, 1933, using the Thunberg technique; Peters and Sinclair, unpublished, using measurement of oxygen uptake]. Also, Meiklejohn [1933] could find no extra disappearance of lactic acid corresponding to the extra respiration produced by the addition of the concentrates to the polyneuritic tissue. It was therefore important to study the R.Q. of the tissue under these conditions. A preliminary account of these experiments has already appeared [Sinclair, 1933].

The respiratory quotient of brain tissue from various animals has usually been found to be nearly unity. Loebel [1925] found that the R.Q. of excised rat brain was 0.99 in fructose, 0.92 in glucose and 0.86 in Ringer solution. Dickens and Greville [1933] reported that the R.Q. of brain cortex of rats and rabbits, buffered either with phosphate or bicarbonate, was unity whether glucose, fructose or no substrate was present. Ashford and Holmes [1931] found that the R.Q. of chopped rabbit brain, in Ringer-phosphate (apparently unbuffered), was 0.88 and rose to unity when lactate was added. Himwich and Nahum [1929] analysed the gases in the blood entering and leaving the brains of dogs and obtained values of unity under a variety of conditions. Himwich and Fazikas [1932] stated that minced tissue of rats, in glucose-phosphate solution, gave the following values: cortex 0.99, stem 0.93, cerebellum 0.89, medulla 0.89. All this work has emphasised that the metabolism of mammalian brain under a variety of conditions is essentially that of carbohydrate.

Very recently Himwich *et al.* [1933] have reported observations on the R.Q. of the cerebral cortex of three pigeons and one dog suffering from polyneuritis. The values obtained with the avian tissue, using the Warburg apparatus, were 0.76, 0.86 and 0.66, but it is not stated in what medium the determinations were made. Himwich assumes, apparently on no evidence, that the R.Q. of

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normal avian cortex would be unity and believes that his results indicate a diminished ability of the brain to oxidise lactic acid. Meiklejohn [1933] could demonstrate no inability of the brain to oxidise lactic acid in avian polyneuritis.

Many authors have studied the R.Q. of polyneuritic animals, with very conflicting results. Ramoino [1915] found that when four pigeons were fed on polished rice, their weight and R.Q. fell progressively although their oxygen uptake was not decreased. On feeding an extract of the rice-polishings, their weight and R.Q. increased, and they became very active. The lowest value of the R.Q. was 0.35, and he concluded that the low values were due to incomplete oxidation processes producing toxic substances. Caridroit [1922] reported that the R.Q. of three pigeons fed on a vitamin B-deficient diet started at 0.9, fell to 0.7 and then rose to 0.8 at the time of symptoms. Anderson and Kulp [1922] did careful work on six hens and concluded that there was no noticeable change in R.Q. in vitamin starvation. They concluded that Ramoino's results were due to leaks in his apparatus. Magne and Simonnet [1922] rightly believed that their experiments were too few to analyse the metabolic disturbance in polyneuritis. Nevertheless they concluded that the R.Q. of pigeons was the same in polyneuritis as in fasting. They found that intravenous injection of glucose raised the R.Q. of a fasting pigeon but produced no significant rise in a polyneuritic pigeon, whence they concluded that the latter was "un animal alimenté" but unable to utilise carbohydrates. In contrast to this, Mattill [1923] found no difference in the basal quotients of normal and avitaminous rats and concluded that vitamin B was not specifically related to the metabolism of carbohydrate since in both groups of animals the R.Q. rose after administration of sucrose or glucose. Gerstenberger and Burhans [1922] believed that studies of the R.Q. showed that polyneuritic pigeons could burn carbohydrates completely; van Berkhout [1926] found a decreased production of CO_2 . Jansen and Mangkoewinoto [1920] generally found a fall in R.Q. in paddy-birds. Okada and Sakurai [1926] and Fleming [1923] reported that the R.Q. was unaltered in human beriberi, and Lawrow and Matzko [1926] actually found a rise in R.Q. and in oxygen consumption in avitaminous hens.

EXPERIMENTAL.

The method of Dixon and Keilin [1933] has been used for the determinations, since this is better than the methods of Dickens and Šimer [1930; 1931, 1] and of Warburg [1924].

The determinations were made over a period of 1 to 2 hours upon minced brain tissue in 3.0 cc. mammalian Ringer solution buffered with $M/10 \text{ KH}_2\text{PO}_4$, p_{H} 7.3, and containing sodium lactate (0.033 M) (as described by Passmore *et al.* [1933]). The side-bulbs contained 0.3 cc. 2 N HCl , and the bottom taps 40 % KOH .

The birds were killed by decapitation with a guillotine. The skull was opened, and the brain removed, freed as far as possible from membranes and blood and finely minced with a bone spatula. The tissue was then divided into as nearly equal portions as possible and placed in the previously filled and tared bottles. After re-weighing, the tissue was finely divided with a flat-ended glass rod¹, the rolls of filter-paper and the glass rods were inserted, and the apparatus assembled and placed in the bath at 38°. 50 to 55 minutes elapsed from the time of death until this stage was reached. Oxygen was passed for 10 minutes through the apparatus while shaking

¹ It is found that if care is taken in breaking up the tissue with the glass rod, no tissue is removed from the bottle, and the amount of medium lost is very small and equal to the amount lost from the control bottle.

by the method described by Dixon and Keilin. The gas was then shut off, the side-taps closed and the apparatus shaken for a further 3 minutes. The taps were then closed and the acid tipped into the left-hand bottles. The remaining procedure was as described by Dixon and Keilin.

In the early experiments, mercury was used to displace the KOH but was found to be unsatisfactory as it sometimes shook over into the medium. In most of the experiments glass rods have been used (see Dixon and Keilin). The apparatus have not been shaken at the speed recommended by Dixon and Elliott [1930], because at that speed the tissue becomes washed up and stuck upon the sides of the bottles, and there is danger of the acid not reaching and killing it immediately. A speed of about 70 complete revolutions per minute has been used.

The constants of the apparatus were calculated from the formula given by Dixon and Keilin. When oxygen and phosphate buffer have been used instead of CO₂ and bicarbonate, the changes in pressure have not been large, and so paraffin, coloured with Sudan III, has been used in the manometers.

All determinations, except where stated, have been done in duplicate and a few in triplicate or quadruplicate. When the duplicates have not agreed to within $\pm 5\%$, the average value has been bracketed. I have found that the duplicates agree better for the measurement of Q_{O_2} than for R.Q. This may be due to the tap-grease (made from lard, bees-wax and olive oil) dissolving CO₂. This would, of course, affect the R.Q. but not the Q_{O_2} . The error from this source can only be small and does not apply to the determinations made in presence of CO₂.

Throughout, precisely the same additions have been made to the left-hand bottle as to the right-hand, but it was found impossible to get the weights of tissue identical. In all experiments, the amounts used have been between 90 and 200 mg., and the amounts in any pair of bottles have always agreed to within $\pm 5\%$, usually considerably less. The differences in bound CO₂ will therefore not be significant.

The birds used in nearly all the experiments have been either Blue Chequer or Blue Bar Homer pigeons. About equal numbers of each sex have been used, but as neither the type of bird nor the sex appears to make any significant difference to the results details have been omitted. In most of the experiments, cerebrums only have been used. Although the lesion in polyneuritis is more marked in the lower parts of the brain [Kinnersley and Peters, 1930], the cerebrum provides more tissue, a higher Q_{O_2} and a more homogeneous mince. Varying amounts of white matter are unavoidably included, but two experiments have shown that the R.Q. of the cerebrum is not very different from that of the lower parts of the brain.

RESULTS.

Table I shows some results obtained with normal pigeons [fed on mixed corn, as described by Kinnersley *et al.*, 1928].

Table I. *Normal tissue in phosphate-buffered lactate.*

Exp.	Tissue	Time (mins.)	Q_{O_2}	R.Q.	
2. iii. 33	Whole brain (except cerebellum)	60	—	0.89*	
3. iii. 33	"	60	—	0.90†	
6. iii. 33	"	60	—	0.86	
13. iii. 33	"	60	8.63*	0.88†	With addition of 2γ vitamin B ₁ per 3.0 cc.
28. iii. 33	"	60	7.35†	0.83†	
26. iv. 33	{ Cerebrum	45	(8.60)	0.93	
	{ Optic lobes and mid-brain		5.75	0.92‡	
22. iv. 33	Cerebrum	60	(10.73)	0.87	
15. viii. 33	"	90	10.73	0.99‡	Q_{O_2} R.Q.
16. viii. 33	"	60	9.05	0.83	10.53 0.96‡
18. viii. 33	"	90	9.45‡	0.88‡	9.80‡ 0.90‡
	"				8.60 0.90

* Quadruplicate.

† Triplicate.

‡ Single determination.

Table II shows results obtained with tissue from birds killed while in opisthotonus due to deficiency of vitamin B₁. The birds had been fed for about 30 days on polished rice by the usual methods used in this laboratory, and the precautions of Kinnersley *et al.* [1928] were observed. The vitamin preparation used was a crystalline concentrate (No. 64-19 of Kinnersley *et al.* [1933]). The bottles to which it was to be added received only 2.0 cc. of the ordinary medium. After the tissue had been broken up with the glass rod, the vitamin was added made up in 1.0 cc. of the medium. (The addition was made to both left- and right-hand bottles.) The amount used (1 to 2 γ per 3.0 cc.; 2.2 γ =1.0 International unit) was such as would give an almost maximum catatorulin effect [Passmore *et al.*, 1933].

Table II. *Avitaminous cerebrums in lactate.*

Exp.	Time (mins.)	Q_{O_2}		R.Q.		R.Q. of extra respira- tion	Vitamin conc. (γ /3.0 cc.)
		Control	Vitamin	Control	Vitamin		
10. v. 33	60	5.50	6.15*	0.74	0.84*	1.7	2.2
11. v. 33	130	(8.70)	11.00	0.65	0.88*	1.3	2.2
6. vi. 33	63	5.80	7.18	0.62	0.89	2.0	2.0
9. vi. 33	60	7.50	8.85*	0.74	—	—	1.0
12. vi. 33	120	4.35	5.58	0.69	0.87	1.5	2.0
16. vi. 33	60	5.25*	7.80	0.58*	0.77	1.2	2.0
19. vi. 33*	60	5.35	6.55	0.71	0.93	1.9	2.0
12. vii. 33	120	4.23	6.20	0.68	(0.91)	1.4	2.0

* Single determination.

Table III shows some results obtained with birds fed on polished rice for varying periods. In some cases, symptoms were threatening; in others, the bird was very active and apparently in good health. It will be seen that in some cases there is a slight rise in the R.Q. and Q_{O_2} , in others no effect.

Table III. *Cerebrums of rice-fed birds in lactate.*

Exp.	Time (mins.)	Q_{O_2}		R.Q.		No. of days on diet	% loss in wt. on diet
		Control	Vitamin	Control	Vitamin		
27. iii. 33	60	5.95*	—	0.67	—	19	—
20. vi. 33†	65	9.05	—	0.86	—	20	—
3. vii. 33*	60	9.20	10.15	0.81	0.87	26	35.6
6. vii. 33*	60	9.00	9.40	0.88	0.75	22	33.2
7. vii. 33*	60	8.00	7.80	0.99	1.09	23	32.3
10. vii. 33	60	8.98	9.10*	0.76	(0.83)	26	36.5
11. vii. 33	60	8.15*	8.08	0.81*	1.01	27	37.0
13. vii. 33	90	7.28	8.03	0.92	0.91	21	26.6

Vitamin concentration, 1 γ /3.0 cc. in Exp. 6. vii. 33; 2 γ /3.0 cc. in the remainder.

* Single determination.

† Triplicate.

Determinations in CO₂-bicarbonate.

Experiments in a medium containing $M/10$ KH₂PO₄ cannot be regarded as being very physiological. Yet Dickens and Šimer [1931, 2] found that the respiration of normal tissue with glucose as substrate was affected neither qualitatively nor quantitatively by substituting CO₂-bicarbonate for phosphate. Ashford and Holmes [1931], however, found that the oxygen uptake of chopped rabbit brain, both in presence and absence of lactate, was larger in CO₂-bicarbonate than in phosphate, and the value of the "Meyerhof quotient" also de-

pended upon the buffer used. Meyerhof [1925] found that phosphate was necessary for the resynthesis of lactic acid in muscle; and Meyerhof *et al.* [1925] showed that there were quantitative differences in respiration in presence of sodium or of potassium phosphate. It was therefore important to study the respiration of the avitaminous brain in CO₂-bicarbonate, with and without added phosphate.

The experiments have been performed in Ringer-lactate (as above) with 0.025 *M* sodium bicarbonate. The final solution when saturated with CO₂ had *p*_H 7.3, but practically no calcium was precipitated. In the experiments with phosphate, sodium dihydrogen phosphate was added to make the final concentration *M*/45. This was brought to *p*_H 7.3 with 20 % NaOH. Gas mixture composed of O₂ + 5 % CO₂ was used, and the medium was saturated with this before being added to the bottles. The manometers were filled with Clerici fluid. Variations in the tonicity of the various media have not been controlled, since Gavrilescu and Peters, in unpublished observations, found that large variations produced no effect upon the respiration.

Tables IV and V show the results obtained with tissue from normal and avitaminous birds respectively.

Table IV. *Normal tissue in CO₂-bicarbonate with lactate.*

Exp.	Tissue	Time (mins.)	Medium	Q _{O₂}	R.Q.	Q _M ^{O₂}
12. ix. 33	Cerebrum	120	Lactate	5.90	0.83	-1.1
13. ix. 33	Whole brain (except cerebellum)	120	Lactate	6.03*	0.81†	-1.2†
15. ix. 33	"	120	Lactate	5.55	0.79	-0.8
			Lactate + phosphate	6.15	0.90	(-1.1)
18. ix. 33	"	120	Lactate	6.53	0.82	-1.2
			Lactate + phosphate	6.50‡	0.93‡	(-1.7)‡

* Quadruplicate.

† Triplicate.

‡ Single determination.

Table V. *Avitaminous tissue in CO₂-bicarbonate with lactate.*

Exp.	Time (mins.)	Medium	Q _{O₂}	R.Q.	Q _M ^{O₂}	R.Q. of extra respira- tion
19. ix. 33	120	Lactate	4.98	0.71	-0.5	—
		Lactate + vitamin B ₁	6.20	0.81	-0.8	1.2
21. ix. 33	109	Lactate	5.68	0.72	-0.4	—
		Lactate + vitamin B ₁	6.45	0.80	-0.7	1.5
16. ix. 33	110	Lactate + phosphate	3.70*	—	—	—
		Lactate + phosphate + vitamin B ₁	5.28	0.91	(-1.0)	2.0
20. ix. 33	120	Lactate + phosphate	4.38*	0.75*	(-0.8)	—
		Lactate + phosphate + vitamin B ₁	5.65	0.88	(-1.3)	1.5

Tissue. Whole brain except cerebellum.

Vitamin B₁, 2γ/3.0 cc.

* Single determination.

Table VI gives the average values obtained in the whole series of experiments. After the mean value for each series is given the standard deviation, $\sqrt{\frac{(\bar{x} - x)^2}{n-1}}$; "t" is the difference between two means divided by the estimated standard error of this difference; and "P," the probability of falling outside the range $\pm t$, is derived from the Table given by Fisher [1932].

Table VI. *Mean values \pm standard deviation.*

A. Phosphate:	R.Q. $\pm \sigma$	"t"	"P"	R.Q. of extra respiration
Normal brain (except cerebellum)	0.87 \pm 0.05	1.22	0.23	—
Normal cerebrum	0.90 \pm 0.06			
Normal cerebrum + vitamin B ₁	0.92 \pm 0.03			
"Rice-fed" cerebrum	0.86 \pm 0.09	1.03	0.33	—
"Rice-fed" cerebrum + vitamin B ₁	0.91 \pm 0.11			
Avitaminous cerebrum	0.68 \pm 0.06	71	0	1.6 \pm 0.3
Avitaminous cerebrum + vitamin B ₁	0.86 \pm 0.06			
B. CO ₂ -bicarbonate:	R.Q. $\pm \sigma$	Q _M ^{0.5}		R.Q. of extra respiration
Normal brain (except cerebellum)	0.81 \pm 0.02	- 1.1		—
Ditto + phosphate	0.91 \pm 0.05	(- 1.3)		
Avitaminous brain (except cerebellum)	0.71 \pm 0.02	- 0.45		1.4 \pm 0.2
Ditto + vitamin B ₁	0.81 \pm 0.02	- 0.75		
Ditto + phosphate	0.75	(- 0.8)		1.8 \pm 0.4
Ditto + phosphate + vitamin B ₁	0.90 \pm 0.05	(- 1.15)		

In calculating the statistics, certain points arise. The standard deviation (σ) has been calculated from the whole set of values in each series and not from the average values (mean of duplicates, *etc.*) given in Tables I to V. In two experiments, only half the usual concentration of vitamin was used (1 γ instead of 2 γ), but as already stated 1 γ is known to give a nearly maximum catatorulin effect. In the case of the birds fed for different periods on polished rice, but not in opisthotonus, the standard deviation is very large because the birds were in varying degrees of vitamin deficiency. The experiments performed with bicarbonate buffer have been too few to permit of convincing conclusions being drawn.

Fisher's method of testing the significance of a difference between two means has been used, although the same results can of course be obtained from the direct application of the probable error concept. The analysis shows that the difference between the R.Q. of avitaminous cerebral tissue with and without added vitamin is undoubtedly significant; the difference between the R.Q. of normal brain and normal cerebrum is probably significant; between the R.Q. of cerebral tissue of rice-fed birds, with and without added vitamin, there is probably no significant difference; and the addition of vitamin to normal cerebral tissue does not significantly change the R.Q.

DISCUSSION.

Many authors have claimed that vitamin B₁ is not specifically related to tissue oxidation processes, but that the results obtained have been due to the loss in weight or general inanition of the animals studied [*e.g.* Drummond and Marrian, 1926; Galvao and Cordoso, 1932; Abderhalden and Vlassopoulos, 1931]. Meiklejohn *et al.* [1932] proved that vitamin B₁ deficiency and inanition were independent, but Westenbrink [1932] has revived the controversy. The results presented here show that the minced avitaminous tissue has a decreased R.Q. as well as a lowered respiration, and that the addition of minute amounts of vitamin B₁ *in vitro* raises both nearly to the normal values. Further, the vitamin has no effect upon the normal tissue and probably no significant effect upon the tissue of birds fed with polished rice but not showing the symptoms of polyneuritis. The results obtained therefore lend strong support to the conclusions of Peters and his colleagues.

These results can be interpreted in many ways. Taking the simplest explanation and assuming that no protein is being oxidised¹, the R.Q. of 0.9 for the

¹ The aerobic production of ammonia by brain is well established [Loebel, 1925; Schwarz and Dibold, 1931; Riebeling, 1931]. Any such production will affect the values neither of the R.Q. nor of the oxygen uptake.

normal tissue may be taken to represent a respiration of which 66 % is carbohydrate and 34 % is lipid. It is interesting to recall that Peters and Sinclair [1933] found that rather more than 60 % of the respiration was sensitive to cyanide or fluoride. The avitaminous tissue has an R.Q. of about 0.68, but it is unlikely that this represents an oxidation of fat alone since lactate markedly stimulates the respiration of this tissue (although not to the same extent as with the normal) and since Meiklejohn [1933] found that the avitaminous tissue readily removed lactate. It is more probable that an incomplete oxidation is occurring in the avitaminous tissue whereby some substance is produced which is only oxidised in presence of the vitamin, thus producing the apparent R.Q. of 1.5 of the extra respiration induced by the vitamin.

The fate of the lactic acid is indicated by the experiments in bicarbonate. The aerobic glycolysis can be measured in these.

The figures given for the $Q_M^{O_2}$ in presence of phosphate do not represent true values and so have been placed in brackets (see Tables IV, V and VI). The apparatus does not give a direct measure of the $Q_M^{O_2}$ if phosphate is present, since buffering by the phosphate minimises any changes in the acid content of the medium. Therefore the true value for the disappearance of acid (negative $Q_M^{O_2}$) in presence of phosphate should be very much larger than the figure given in the Tables.

The removal of acid in the avitaminous brain in absence of phosphate is found to be very small ($Q_M^{O_2} = -0.45$), but upon adding vitamin B₁ there is a large increase ($Q_M^{O_2} = -0.75$) approaching the value for the normal tissue ($Q_M^{O_2} = -1.1$). There is a corresponding rise in the respiration and in the R.Q. Yet the latter is only 0.81 (for the normal tissue, and avitaminous + added vitamin), and the R.Q. of the extra respiration produced by the vitamin is about 1.4. Therefore it seems improbable that the increased disappearance of acid merely indicates an oxidation of lactic acid.

Adding phosphate hardly affects the oxygen uptake either of normal or of avitaminous brain with added vitamin, but raises the R.Q. from 0.81 to 0.91 (representing an increase of 35 to 69 % in the carbohydrate part of the respiration). Also, the disappearance of acid is greatly increased¹. But since this large disappearance can hardly all be accounted for by the increase in the carbohydrate part of the respiration, it is simplest to regard it as a synthetic removal of lactic acid. Speculation, however, would not be justifiable.

SUMMARY.

1. The respiration of brain tissue from normal, rice-fed and polyneuritic pigeons has been studied in presence of lactate buffered with either phosphate or bicarbonate.
2. The respiratory quotient of the tissue from polyneuritic pigeons is low and is raised nearly to the normal value by the addition *in vitro* of minute amounts of crystalline vitamin B₁.
3. The low value is independent of inanition, since the addition of vitamin does not influence the R.Q. of tissue from normal pigeons and hardly influences the quotient of tissue from pigeons fed on polished rice but not showing symptoms.
4. The specific effect of vitamin B₁ in raising the respiration of avitaminous brain tissue is found in lactate buffered with bicarbonate, and there is also a

¹ It is improbable that the negative aerobic glycolysis is due in any large degree to production of ammonia or breakdown of creatinephosphate, since it is unlikely that either would be greatly increased by the addition of phosphate.

rise in the respiratory quotient. These results are independent of the addition of inorganic phosphate to the medium.

5. The aerobic glycolysis of the avitaminous tissue is slightly negative and becomes more so when vitamin B₁ is added or with normal tissue. The addition of phosphate markedly increases this negative value.

I am very deeply indebted to Prof. Peters for his constant interest and advice throughout this work. I also wish to thank Mr Kinnersley for the supply of the vitamin B₁ concentrate and Dr Malcolm Dixon for early information concerning the apparatus.

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CCLXIII. POLYPLOIDY AND VITAMIN C.

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(*Received November 2nd, 1933.*)

In this communication, evidence is produced which lends strong support to the view that there exists a connection between the polyploid constitution of the plant and its vitamin C content. The experiments to be described were embarked upon as a consequence of some suggestive information which was obtained in a series of investigations on the apple. Some years ago it was observed by Zilva and his collaborators in the course of a comprehensive investigation on the influence of the physiological condition of the apple on its vitamin C content [Bracewell *et al.*, 1930, 1, 2] that the antiscorbutic potency of the Bramley's Seedling variety was very much greater than that of a number of other varieties examined. The striking nature of this observation was stressed by the writers at the time. Attention was then drawn by Prof. J. B. S. Haldane and later by Dr J. T. Bregger, Extension Horticulturist of the State College of Washington, Pullman, Washington, to the fact that the Bramley's Seedling was the only variety amongst those investigated which was a triploid (51 chromosomes in the somatic tissue), whilst the others were diploid varieties (34 chromosomes in the somatic tissue). Acting on this suggestion, Crane and Zilva [1931; 1932] made a comparative study of a number of triploid and diploid apples. They found that in both forms there was a variation in antiscorbutic activity amongst the varieties tested, but that the most active apples fell within the triploid category. The striking feature, however, was that certain triploid fruits showed as low a vitamin C content as some of the least active diploid apples and conversely that certain diploid varieties were as active, if not as the most active triploid apples, at least as some of the moderately active fruits of this category. In interpreting the results it was, therefore, difficult to arrive at a clear-cut conclusion. Crane and Zilva, nevertheless, considered the evidence as favouring the theory that a higher vitamin C content was associated with a higher number of chromosomes in the somatic tissue in the apple.

It is plain that conclusive evidence could only be obtained by comparing the polyploid and the diploid form from which the former was derived. Such comparison could not conveniently be made in the case of the apple. Crane and Zilva therefore suggested that fruits in which polyploidy could be induced with ease should form the future experimental material. In consequence the present authors chose the tomato as a starting-point.

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THE EXPERIMENTAL MATERIAL.

The normal tomato is a diploid with 24 somatic chromosomes, but it is found that when this form is decapitated, about 7 % of the adventitious shoots which arise in consequence of this treatment are tetraploids with 48 somatic chromosomes.

The contrasting characters used in this experiment were tall (*D*) and dwarf (*d*), red-fleshed fruit (*R*) and not red-fleshed (yellow) fruit (*r*). Through linkage phenomena the tall plants were also of the constitution *O* (round fruit), *P* (smooth fruit-skin) and the dwarfs were *o* (oval fruit) and *p* (rough skin).

The above diploid stocks of the genetic constitutions *DOPR*, *dopr*, *DOPr* and *dopR* were induced to form tetraploid shoots by the above method discovered by Winkler [1916] and developed by Jørgensen and Crane [1927]. Self-fertilisation of the diploid and tetraploid plants thus obtained therefore produced progeny of comparable constitutions in these four genetic categories, *i.e.* tall red, dwarf yellow, tall yellow and dwarf red. All these characters are known to be inherited according to Mendelian laws [*cf.* Lindstrom, 1925; 1932; McArthur, 1931]. The production of the experimental material is presented diagrammatically in Fig. 1.

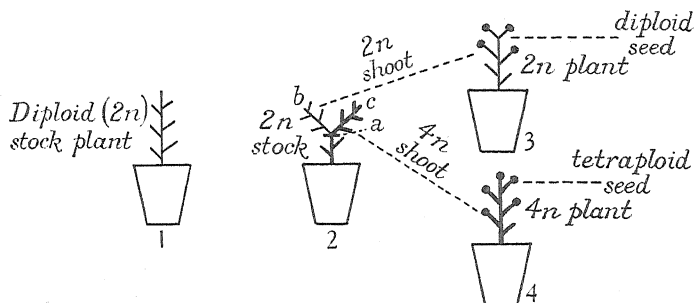


Fig. 1. 1, Normal diploid ($2n$) tomato (24 chromosomes). 2, Diploid cut back at *a* giving rise to shoot *b* (diploid) and shoot *c* (tetraploid). 3, Diploid plant raised vegetatively from shoot *b* of 2. 4, Tetraploid plant raised vegetatively from shoot *c* of 2. Seeds from 3 and 4 supplied respectively diploid and tetraploid material for this investigation.

THE VITAMIN C TESTS.

The comparison of the vitamin C contents was carried out on the juice of the tomatoes, since the utilisation of the intact fruit might have yielded misleading information owing to variations in the juiceless pulp. Preliminary experiments carried out in 1932 revealed that in order to obtain truly comparable results several precautions had to be taken in carrying out the tests. One of the chief of these was against the instability of the vitamin in the juice. Very marked losses were recorded when the tomato juice was stored even at -20° for any length of time, a temperature at which the activity of many fruit tissues and juices is moderately stable. Further complications arising out of a difference in stability of the vitamin in juices originating from fruits of different constitutions could also not be excluded. As a result of several trials, the following procedure was finally adopted. The fruit was picked from plants growing in adjacent rows at Merton twice a week. The juice was prepared daily immediately before its administration to the experimental animals. The tomatoes were averaged as to size, as far as possible, and also as to degree of ripeness, before

being placed in a refrigerator at -20° the day previous to the preparation of the juice. To each 50 g. of the frozen tomato 20 g. of sand were added and the mixture finely ground. After squeezing through muslin, the residue was well ground again. The squeezed out juice was then added to the ground residue, ground once more and again pressed out through muslin. After centrifuging, the resulting yellow juice was administered to the animals without delay. A yield of juice equivalent to 80 % or more of the original weight of the tomato is thus obtained and the entire procedure does not take more than 30 minutes. The details of the biological test method have been given in previous communications.

Chemical data.

Owing to the limited quantity of experimental material at our disposal we were unable to examine it in great detail. This we hope to do when more material is available. We have nevertheless obtained a certain amount of information in this connection about the juices tested which has some bearing on this investigation.

H ion concentration. This was daily carried out colorimetrically, immediately after the preparation of the juice. There was little variation in the acidity of the juices. Those from diploid tomatoes showed a p_H value of 4.2-4.4, very exceptionally 4.6. The tetraploid juices were slightly more acid, about p_H 4.0. The acidity of both forms was probably due to the presence of weak organic acids since an appreciable quantity of ammonia was required to bring the solutions to p_H 7.0.

Indophenol-reducing capacity. As has been usual for a number of years in this laboratory, the juices were titrated daily with indophenol (dimethylamino-phenylindophenol) just before they were administered to the experimental animals. In these tomato juices, although it would be unsafe to generalise, there is some proportionality between the capacity of reducing this indicator and the antiscorbutic activity. It is therefore of interest to examine the titration results. Space does not permit citation of all the figures, but in Table I there are pre-

Table I. *Titration cc. N/1000 indophenol per cc. of juice.*

Tetraploids				Diploids			
<i>dRop</i>	<i>DRop</i>	<i>drop</i>	<i>DrOP</i>	<i>dRop</i>	<i>DRop</i>	<i>drop</i>	<i>DrOP</i>
3.3	3.2	3.1	3.2	2.5	2.3	2.8	2.0
4.1	4.5	3.5	4.5	1.7	1.6	2.4	1.9
4.5	4.8	4.6	4.9	2.0	2.0	2.7	2.0
4.8	4.9	4.5	4.5	1.6	1.7	1.9	1.6
4.1	5.3	4.7	4.7	1.7	2.6	2.6	2.3
4.2	4.7	4.3	4.1	2.0	2.3	2.6	2.6
3.9	3.0	3.4	3.1	2.9	3.3	3.4	3.2
Average for test:							
4.1	4.3	4.0	4.1	2.0	2.3	2.6	2.2

sented the weekly averages and the averages for the entire test. These figures are representative since there was little variation and consequently not much deviation from the average in the reducing capacity of the individual juices from day to day; extreme figures were obtained only rarely.

If it were assumed that the entire reducing capacity were due to ascorbic acid and that none of the "reversibly oxidised" form of the compound were present the tetraploids would contain 35 mg. and the diploids 20 mg. per 100 cc.

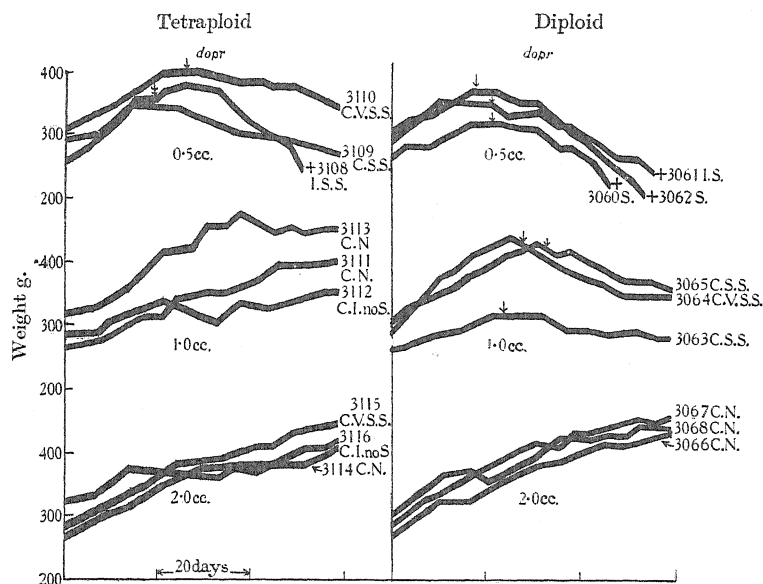


Fig. 2.

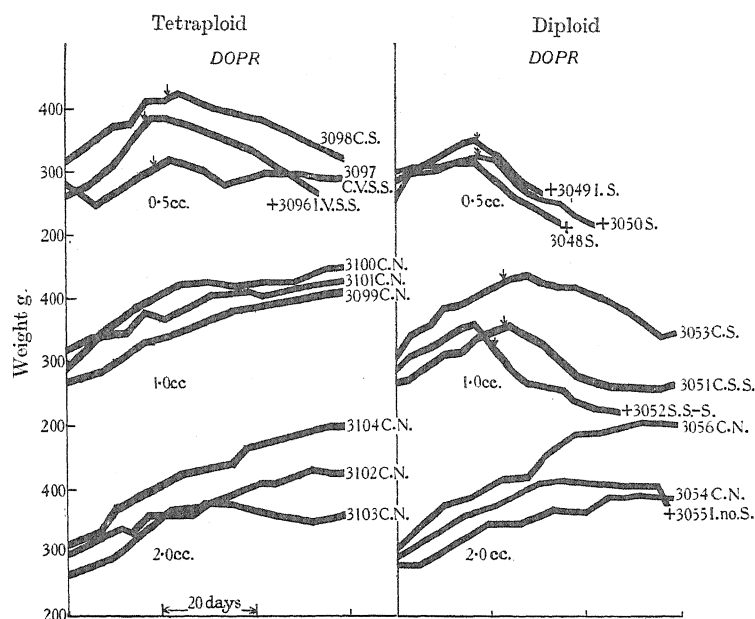


Fig. 3.

C. = chloroformed
 + = died
 S. = scurvy
 S.S. = slight scurvy

V.S.S. = very slight scurvy
 N. = normal
 no S. = no scurvy
 I. = intercurrent infection

↓ = onset of clinical symptoms of scurvy.

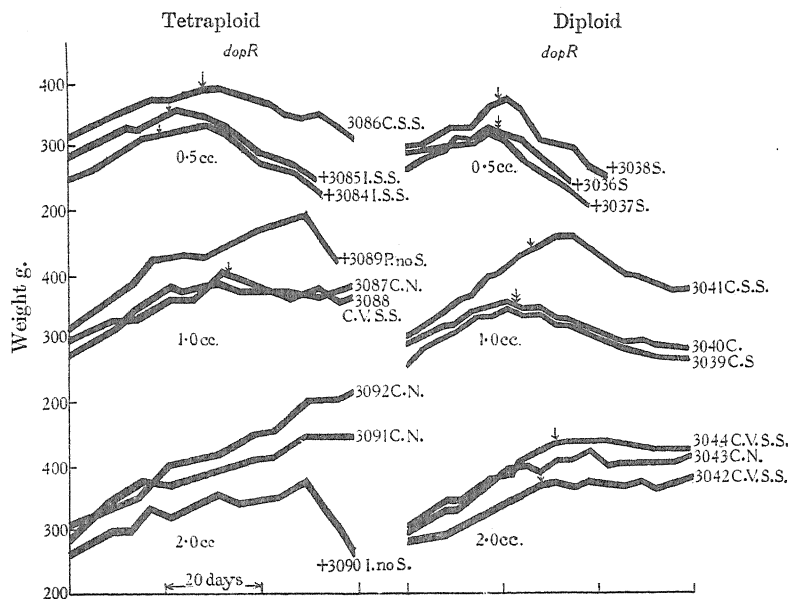


Fig. 4.

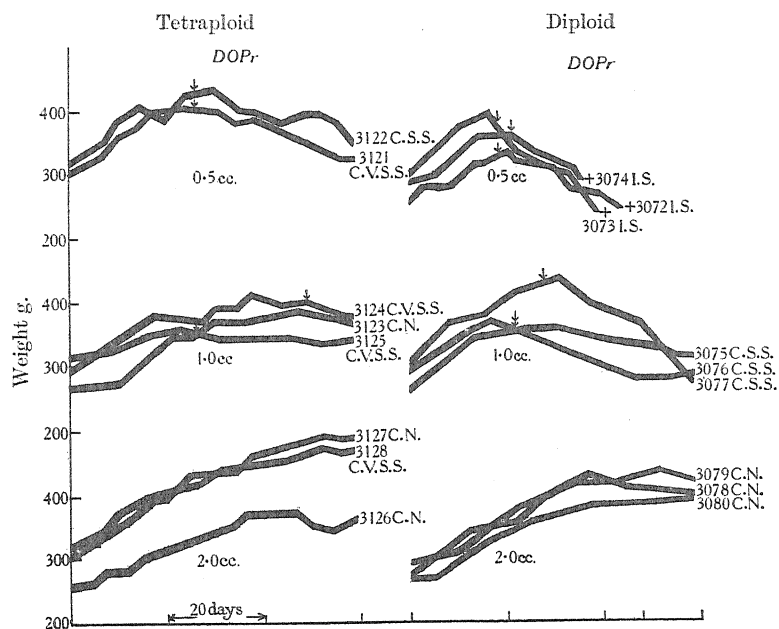


Fig. 5.

C = chloroformed
 + = died
 S = scurvy
 S.S. = slight scurvy
 ↓ = onset of clinical symptoms of scurvy.

V.S.S. = very slight scurvy
 N. = normal
 no S. = no scurvy
 I. = intercurrent infection

of juice. It will be seen later that the biological result was of a similar order in each case.

Dry matter. These estimations were not carried out daily but on several occasions during the progress of the test. The figures are given in Table II. The clear-cut nature of the results is obvious. The juices from the tetraploid varieties contained in every case more dry matter than those from the diploid varieties. It is hoped to continue the study of the chemical nature of the juices when more material is available.

Table II. *Dry matter* %.

Tetraploids				Diploids			
<i>dRop</i>	<i>DROP</i>	<i>drop</i>	<i>DrOP</i>	<i>dRop</i>	<i>DROP</i>	<i>drop</i>	<i>DrOP</i>
6.97	6.76	7.30	8.25	4.49	4.41	4.12	—
—	—	6.71	7.22	—	—	4.39	—
7.82	6.88	4.94	5.10	3.92	3.86	4.15	4.24
8.14	7.94	5.93	7.66	3.34	3.95	4.40	4.53
7.94	5.37	5.92	5.15	4.56	4.27	5.06	4.07
Average:							
7.72	6.74	6.16	6.67	4.08	4.12	4.42	4.28

Biological results.

These results are presented in Figs. 2, 3, 4 and 5 which give weight curves, clinical symptoms of scurvy and *post mortem* details. The results are straightforward and need hardly any comment. In every case the potency of juice from tetraploids is about twice as great as that from diploids and the activity in each case appears to be independent of the genetic characters of the varieties tested. The diploid variety *dopr* may be somewhat more active than the remaining diploid varieties. The corresponding tetraploid form, however, does not seem to possess a higher vitamin C content than the other tetraploids.

Interpreting the above results in terms of International Units and of ascorbic acid, the following data are obtained. In the case of the tetraploids, 1 cc. of juice gave rather better protection than is usually obtained with 1 cc. of decitrated lemon juice and was about equal in potency to 0.5 mg. of ascorbic acid [Hirst and Zilva, 1933]. It accordingly contains rather more than 1000 I.U. or about 50 mg. of ascorbic acid per 100 cc. of juice. The diploid juices on the same basis of assessment contain somewhat more than 500 I.U. or about 25 mg. of ascorbic acid per 100 cc. of juice.

It may be noted here that 1 mg. of ascorbic acid has always so far been observed to be rather more active than 2 cc. of the average decitrated lemon juice (20 I.U.) in this laboratory. As there is no significant variation in the response of the experimental animals in tests performed as above at different times, it may be safely assumed that this equivalence always holds true.

DISCUSSION OF RESULTS.

The results presented in this communication leave little doubt that a connection exists between polyploidy and vitamin C content in the tomato, which further strengthens the view of Crane and Zilva that a similar relationship exists also in the apple. This raises some interesting speculations concerning the latter fruit. It has already been mentioned that one of the diploids (Lane's Prince Albert) was as active as the moderately active triploid apples. Is it possible that the very active triploid apples were derived from a diploid strain or strains with a similarly high antiscorbutic activity? Conversely, is the origin

of the triploid Gravenstein with a very low vitamin C content to be traced to a diploid form with an exceptionally low antiscorbutic potency?

The genes investigated in this research seem to have no bearing on the vitamin C content of the tomato. It is not, however, excluded that other factors, whether genetic or not, may exercise such an influence. In fact we have obtained indications to this effect. For instance the wild tomato, *S. racemigerum*, which is a diploid shows a higher titration value with indophenol than do any of the tetraploid varieties of *L. esculentum* so far investigated. If the indophenol-reducing capacity should prove a reliable index of the antiscorbutic activity in this case, the higher vitamin content of this variety must be due to genes rather than the number of chromosomes.

The fruits from the tetraploid tomato plants are smaller than those from diploid plants but it is hardly likely that the higher concentration of vitamin C in the juice of tetraploids is indirectly due to the size of the fruit. We found that larger fruits showed if anything a higher indophenol-reducing capacity than the smaller ones belonging to the same plant and as was mentioned in this case the titration value was a fairly good indication of the antiscorbutic potency of the juice.

Reference must here be made to results recorded by Key [1933]. She examined one strain of each of a diploid and tetraploid tomato of comparable constitution for its vitamin C content by the tooth method and found no difference between their antiscorbutic activities. Her results show further that the vitamin C content of the above strains of tomato and of a sample bought in the open market was much lower than that recorded by us. We are unable to explain these divergencies.

SUMMARY.

The tetraploid and diploid forms of the strains of the tomato, *DOPR*, *dopR*, *dopr*, *DOPr*, obtained by the induction of polyploid method and subsequent vegetative propagation were examined for their vitamin C content by the prophylactic test. All the tetraploid strains were found to be about twice as active as the diploids. The tetraploids contained rather more than 1000 International Units or 50 mg. of ascorbic acid per 100 cc. of juice.

The above results strengthen the evidence obtained by Crane and Zilva that in the case of apples there is a connection between the vitamin C content and the number of chromosomes.

There is no association between the genetic factors *DR*, *dR*, *Dr* and *dr* and the vitamin C content of the tomato.

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CCLXIV. THE INDOPHENOL-REDUCING CAPACITY AND THE VITAMIN C CONTENT OF EXTRACTS OF YOUNG GERMINATED PEAS.

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(Received November 6th, 1933.)

It has been known for a considerable period that though many dried seeds are devoid of antiscorbutic activity, they acquire this property very readily on germination. This observation, first made by Young [1782; quoted by Curtis, 1807], was demonstrated in the laboratory by Fürst [1912]. Quantitative data in this connection were first provided by Chick and her collaborators. In the case of peas (*Pisum sativum*) Chick and Delf [1919] found that young growing guinea-pigs could be fully protected from scurvy by adding as a supplement to their scurvy-producing diet 10 g., or in some cases 5 g., of peas which had been germinated for 2 days.

Recently the technique in which the capacity of antiscorbutic solutions for reducing indophenols, introduced by Zilva [1927], has been utilised by several workers in the quantitative assessment of vitamin C, since this vitamin (*l*-ascorbic acid) is known to reduce these indicators. This procedure is open to grave objections not only because substances other than ascorbic acid can reduce these indicators, but also on account of the fact that ascorbic acid is capable of existing in an oxidised form which possesses a very high antiscorbutic activity but which is incapable of reducing indophenols.

Harris and Ray [1933], on the basis of indophenol titrations of trichloro-acetic acid extracts, record the following values for the ascorbic acid content of germinated peas.

	Ascorbic acid content	
	mg. per g.	mg. in single seed or seedling
Seed peas, before germination	0.00	0.00
" soaked 24 hrs. (not germinated)	0.08	0.02
" " 48 " (germinated)	0.69	0.21
" " 72 " (germinated)	0.82	0.26
" " 96 " (germinated)	0.86	0.27

Euler and Klusmann [1932; 1933, 1, 2] state that extracts obtained from dried fresh green peas and from pea seedlings varying in age from 7 to 32 days possess a remarkably low capacity for reducing indophenol. In the case of an extract of a 32-day old seedling this reducing capacity was increased ninefold by treatment with hydrogen sulphide, but even then its value was considerably less than that anticipated from its known biological activity. The spectrophotometric estimation of the ascorbic acid content of the extract before treatment with hydrogen sulphide appeared to show that it contained 100 times the amount of this substance indicated by the indophenol titration. On the basis of these

observations Euler suggests that the ascorbic acid in the germinating seedling is bound to "Hemmungstoffe," presumably in such a manner as to inhibit its reducing properties, from which it is liberated by hydrolysis in the animal body. So far, however, his experiments with various enzymes *in vitro* have failed to substantiate this opinion.

The experiments to be described here were undertaken in order to ascertain firstly whether a quantitative relationship exists between the indophenol-reducing capacity and antiscorbutic activity of extracts of peas in the early stages of germination and secondly, if no such relationship exists, whether this is due to the presence of an active oxidised form of the vitamin in the young seedling. Evidence will be produced which shows that the indophenol-reducing capacity of extracts is no true index of the antiscorbutic potency of the seedlings from which they were obtained and that the formation of the active oxidised form of the vitamin during the early stages of germination is unlikely.

EXPERIMENTAL.

Indophenol titrations of pea extracts.

In all cases, titrations were made with *N*/1000 indophenol (*p*-dimethylamino-phenylindophenol) which was standardised against titanous chloride. Titrations in acid solution were usually carried out by adding 1.0 cc. of 80 % acetic acid to 5 cc. of neutral extract.

I. *Dried peas* (*Pisum sativum*).

The dried peas were ground in a mill and then passed through a sieve to remove the skins. The sieved product was then milled to a fine flour. All the extracts described below were prepared from this flour, typical examples of each series being given below.

(a) *Aqueous extract.* 5 g. of pea flour were ground in a mortar for 10 minutes with 25 cc. of water and the mixture then left to stand for half an hour, after which it was centrifuged. The reaction of the supernatant liquid was adjusted to p_H 7.0 with dilute ammonia. The extract was turbid and yellow-green in colour.

1 g. equivalent of the pea flour reduced 8.0 cc. of *N*/1000 indophenol. The rate of reduction was relatively high at first but slowed down considerably towards the end. Titrations in acid solution were vitiated by the separation of a voluminous precipitate. A similar precipitate also separated on the addition of alkali. The extract did not reduce ammoniacal silver nitrate solution but it gave a marked nitroprusside reaction.

(b) *Phosphate extract.* 5 g. of pea flour were extracted with 25 cc. of 0.87 % K_2HPO_4 solution (Thunberg's solution) in precisely the same manner as the above. This extract was similar in appearance and properties to the aqueous extract.

1 g. equivalent of pea flour reduced 8.0 cc. of *N*/1000 indophenol in a similar manner to the previous extract.

(c) *Trichloroacetic acid extract.* 20 g. of pea flour were extracted in the above manner with 50 cc. of 5 % trichloroacetic acid. The deep green-coloured liquid was adjusted to neutrality with ammonia and centrifuged. A clear light yellow-green extract was finally obtained. 1 g. equivalent of the pea flour reduced 2.5 cc. of *N*/1000 indophenol quickly in acid solution and the same amount rather slowly in neutral solution. The extract gave a nitroprusside reaction.

It also deposited fine sharp-pointed needles on making alkaline with ammonia. It did not reduce ammoniacal silver nitrate solution.

(d) *N/10 sulphuric acid extract.* This extract was obtained from 20 g. of pea flour and 50 cc. of *N/10* sulphuric acid by the same procedure as above. The neutralised extract was similar in character and properties to the trichloroacetic acid extract. 1 g. equivalent reduced 2.5 cc. of *N/1000* indophenol in acid solution and 3.8 cc. at a slower rate in neutral solution.

(e) *Protein-free aqueous extract.* The rather low reducing capacity of the last two extracts seemed to show that this condition was associated with the absence of protein, indeed the removal of protein from aqueous extracts caused a diminution of their capacity for reducing indophenols, as will be seen from the following examples.

20 g. of pea flour were extracted with 50 cc. of water as previously described. In order to free it from protein the extract was acidified with 0.5 cc. 80 % acetic acid and centrifuged. The supernatant liquid was then decanted, heated to boiling-point, cooled and filtered. The filtrate was adjusted to neutrality with ammonia and filtered from the small amount of flocculent precipitate which separated. The final solution was clear and green-yellow in colour. 1 g. equivalent of pea flour reduced only 2.5 cc. of *N/1000* indophenol in both acid and neutral solution. The extract was also similar in properties to the acid extracts.

(f) *Protein-free phosphate extract.* This extract was prepared in the same manner as the previous extract from 20 g. of pea flour and 50 cc. of 0.87 % K_2HPO_4 solution. It had the same character and reducing properties as the aqueous extract and possessed the same capacity for reducing indophenol.

In all these extracts, the intensity of the nitroprusside colour appeared to be proportional to the capacity for reducing indophenol and the amount of the crystalline precipitate obtained with ammonia from the protein-free extracts was always greatest in those which gave the most intense nitroprusside reaction. The presence of a reducing substance other than ascorbic acid, which would reduce indophenol, was anticipated from the work of Kozlowski [1926; 1931] who isolated from peas a compound which contained cysteine and resembled glutathione. Whether the reducing substance met with here is identical with the one which Kozlowski isolated from peas has not been determined, but the crystalline material precipitated from the protein-free extracts by ammonia certainly resembles a similar substance which he found associated with his glutathione-like compound and which evidently can be obtained from the latter by hydrolysis.

II. *Germinated peas.*

The peas were soaked for 24 hours and then spread out on a layer of wet clean silver sand and lightly covered with drier sand so that they could respire freely. After 3 days, the seedlings were taken up, washed free from sand and the skins removed before extracts were prepared from them. The sand was well washed after each germination.

Taking the dry weight of the seed peas as 100 %, the average dry weights of some 30 batches of soaked and germinated seeds were 53 % and 43 % respectively. The average length of the radicles of the same batches of seedlings was 4.2 cm. The variations from these averages were small.

(a) *Aqueous extract.* 25 g. of germinated peas were finely ground in a mortar with 10 g. of clean sand and 50 cc. of water. The residue obtained by squeezing the mixture through muslin was again ground until it was uniformly fine. The watery extract was then added to it and the mixture well ground once more,

after which it was left to stand for half an hour. It was finally squeezed through muslin and the liquid centrifuged. The resulting extract, which was almost neutral (p_H 6.0) was yellow-green in colour and turbid. The addition of acids or alkalis caused the separation of a bulky precipitate. The titrations varied a great deal and only a few of the extracts gave a nitroprusside reaction. None of them reduced ammoniacal silver nitrate in the cold. The average titrations of 35 such extracts were 1.1 cc. and 0.9 cc. $N/1000$ indophenol at acid and neutral reaction respectively per g. equivalent of germinated pea.

(b) *Phosphate extract.* This extract was prepared in the same manner as the above, except that the germinated peas were extracted with 50 cc. of 0.87 % K_2HPO_4 solution. The extract, which was darker in colour than the aqueous extract, invariably gave a nitroprusside reaction and like that extract it did not reduce ammoniacal silver nitrate in the cold. It was also almost neutral in reaction and gave heavy precipitates with acids and alkalis. The indophenol titrations in this case were fairly uniform. The reduction of the indicator, however, was a composite effect which was due to at least two reducing substances. One of these substances, like ascorbic acid, reduced the indicator very quickly in acid and more slowly in neutral solution. The other, which was most probably the substance which gave the nitroprusside reaction (possibly Kozłowski's glutathione compound), reduced the indicator at a slower rate at both acid and neutral reaction. The average titrations due to the rapidly reducing substance or substances of 35 such extracts were 2.65 cc. and 2.5 cc. of $N/1000$ indophenol at acid and neutral reaction respectively per g. equivalent of germinated pea.

(c) *Trichloroacetic acid extract.* 50 g. of germinated peas were extracted with 75 cc. of 5 % trichloroacetic acid by the same procedure as above. After centrifuging, the extract was neutralised with ammonia and filtered. The final solution was light green-yellow in colour and slightly turbid, due probably to the starch which it contained. It invariably gave a nitroprusside reaction and deposited a crystalline substance (sharp-pointed needles) on making alkaline with ammonia, which was identical with that obtained from the dry pea flour. Its ability to reduce ammoniacal silver nitrate was doubtful, it inhibited the reduction of this reagent by decitrated lemon juice.

In the case of this extract, the extent of the reduction of indophenol by the substance or substances which reduced it rapidly was more easily ascertained than in the case of the phosphate extract. 1 g. equivalent of the extract had, thus, an average titration of 2.2 cc. of $N/1000$ indophenol at both acid and neutral reactions.

(d) *Sulphuric acid extract.* 50 g. of germinated peas were extracted with 100 cc. of $N/10$ H_2SO_4 in the same manner as in the case of the trichloroacetic acid extract. The final preparation was clear and contained no starch. Its properties were usually the same as those of the previous extract though it generally gave a more intense nitroprusside reaction than the trichloroacetic acid extract and deposited a greater abundance of the needle crystals on making alkaline with ammonia. 1 g. equivalent of the germinated peas reduced quickly 2.0 cc. $N/1000$ indophenol at both acid and neutral reactions.

(e) *Aqueous cyanide extracts.* It is now well known that the oxidation of ascorbic acid is greatly retarded by the presence of cyanides [Szent Györgyi, 1928; Euler, Myrbäck and Larsson, 1933]. It was, therefore, desirable to examine extracts prepared with solutions containing cyanides. The extract was obtained from 25 g. of germinated peas with 50 cc. of water containing 1 cc. of 5 % NaCN solution by the procedure already described for the simple aqueous

extract. The protein was removed from it with acetic acid in the same manner as in the case of the aqueous extract of dry pea flour. The final preparation was very similar in all its properties to the sulphuric acid extract described above. An average of several titrations showed that 1 g. equivalent of germinated peas rapidly reduced 2.4 cc. and 2.6 cc. *N*/1000 indophenol in acid and neutral solution respectively.

(f) *Cyanide-phosphate extracts.* The procedure followed here was precisely the same as in the previous case. A mixture of 1.0 cc. of 5 % NaCN and 8.5 cc. of 0.87 % K_2HPO_4 solutions diluted to 50 cc. was used to extract 60 g. of germinated peas. These preparations gave a more intense nitroprusside reaction, deposited more crystals on making alkaline with ammonia and had, consistently, a greater capacity for reducing indophenol quickly than the corresponding aqueous extract. The indophenol titrations were quite uniform. The averages for 42 such extracts per g. equivalent of germinated peas were 3.0 cc. and 3.4 cc. *N*/1000 indophenol in acid and neutral solution respectively.

The effect of hydrogen sulphide on various extracts of germinated peas. The presence in these extracts of the active oxidised form of the vitamin, which is incapable of reducing indophenol, was next considered. Attempts were made to convert any such form, if present, to the reduced form by treatment with hydrogen sulphide. Three extracts were used (1) trichloroacetic acid, (2) deproteinised aqueous extract, (3) deproteinised cyanide-phosphate extract. The treatment with hydrogen sulphide was carried out in the manner previously described [Johnson, 1933]. Table I shows the reducing capacities of these extracts before

Table I.

Extract	cc. <i>N</i> /1000 indophenol reduced by 1 g. equivalent			
	Before treatment with hydrogen sulphide		After treatment with hydrogen sulphide	
	Acid solution	Neutral solution	Acid solution	Neutral solution
Aqueous extract	0.0	0.0	0.3	0.3
Trichloroacetic acid extract	1.8	1.8	2.0	2.0
Cyanide-phosphate extract	2.3	2.3	2.5	2.5

and after treatment with hydrogen sulphide. The small increments shown cannot be considered significant.

Distribution of indophenol-reducing substances between cotyledons and radicles of germinated peas. 70 g. of germinated peas were divided into cotyledons and radicles. The former weighed 60 g. and the latter 10 g.

The cotyledons were extracted with 50 cc. of 5 % trichloroacetic acid in the manner already described. The neutralised extract, which was clarified with a small amount of alumina gel, gave a marked nitroprusside reaction and deposited needle crystals with ammonia in good yield.

1 g. equivalent reduced 3.3 cc. of *N*/1000 indophenol quickly in acid solution and 3.5 cc. in neutral solution.

The radicles were extracted with 20 cc. of 5 % trichloroacetic acid in the same manner as the cotyledons.

The final preparation, which was only faintly coloured, gave only a very doubtful nitroprusside reaction, and deposited an exceedingly small amount of needle crystals on adding ammonia. 1 g. equivalent reduced 4.3 cc. and 4.5 cc. *N*/1000 indophenol quickly at acid and neutral reaction respectively.

There appeared to be only one indophenol-reducing substance present in the radicles judging from the decisiveness of the end-points in the titrations and the absence of substances giving a nitroprusside reaction.

It will be seen that though the radicle has a greater indophenol-reducing capacity per g. than the cotyledons, the latter nevertheless possess 5/6 of the total amount of the reducing substances. Whether this ratio represents the distribution of ascorbic acid, can only be decided by future work. It may be that the reduction of indophenol in the case of the extract of the radicle is due only to ascorbic acid but this does not seem to be the case with the extract of the cotyledon.

Biological tests.

It was not possible to examine all the above preparations biologically for vitamin C content. Representative experiments were, therefore, performed which were calculated to shed some light on the quantitative relationship between the biological activity and the reducing capacity.

(1) *Dried peas.* To four guinea-pigs kept on the usual basal diet used in this laboratory, a daily dose of 2.5 g. of ungerminated peas was given in the form of flour. All the animals succumbed in the usual way to scurvy within the usual time.

(2) *Germinated peas.* Daily doses of 1.0, 2.5 and 5.0 g. were tested in this case and the results are given in Fig. 1. Under the conditions of germination

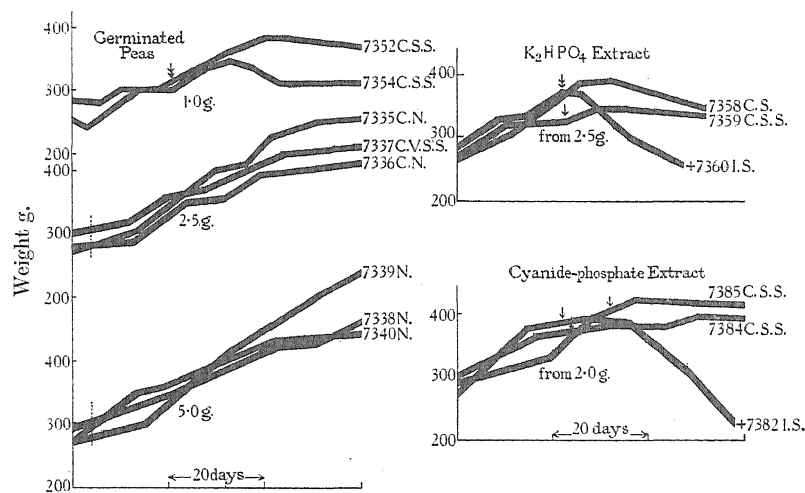


Fig. 1.

C. = chloroformed
+ = died
S. = scurvy
S.S. = slight scurvy
V.S.S. = very slight scurvy

N. = normal
I. = intercurrent infection
... = beginning of dosing
↓ = onset of clinical symptoms of scurvy.

described above the peas were found to contain about 43 % of dry matter. There was no serious deviation from this figure.

(3) *Aqueous extract.* Three guinea-pigs were used in this experiment. A daily dose of an equivalent of 2.5 g. of germinated peas delayed somewhat the onset of scurvy, but all the animals died of the disease between 40 and 45 days. The daily dose of this extract reduced about 2.5 cc. of N/1000 indophenol. A dose

of ascorbic acid of an equivalent reducing capacity (about 0.25 mg.) would have afforded very much better protection [cf. Hirst and Zilva, 1933].

(4) *Phosphate extract.* Daily doses of an equivalent of 2.5 g. of germinated peas were tested on three guinea-pigs and as will be seen from Fig. 1, the protection was very much less than that obtained with 2.5 g. of germinated peas. Furthermore, the average dose reduced about 6.0 cc. of *N*/1000 indophenol which should be equivalent to 0.5 mg. of ascorbic acid. The antiscorbutic potency of this dose, however, falls somewhat but definitely short of that observed with 0.5 mg. of ascorbic acid [Hirst and Zilva, 1933]. The titration value of this extract gives, therefore, no quantitative index of the antiscorbutic potency of the germinated peas from which it was obtained since, in the first place, the vitamin is not thoroughly extracted and, secondly, the reducing capacity of the extract is apparently not entirely due to ascorbic acid. It should be pointed out that these extracts were prepared for the best part of the duration of the test from the germinated peas tested above.

(5) *Cyanide-phosphate extract.* These results are also given in Fig. 1. The average daily dose which was equivalent to 2 g. of the germinated peas reduced about 6.0 cc. of *N*/1000 indophenol. In this case also the equivalent extract was less potent than the germinated peas from which it was obtained and its capacity for reducing indophenol was rather higher than it would have been had the reduction been due to ascorbic acid alone.

DISCUSSION OF RESULTS.

Concerning the first aim of this investigation, namely, to ascertain whether a quantitative relationship exists between the indophenol-reducing capacity and the antiscorbutic activity of extracts of peas and the peas themselves, it may be said that the above results supply fairly conclusive evidence. As was seen, the phosphate and phosphate-cyanide extracts showed only half of the antiscorbutic activity of the peas from which they were obtained. Furthermore, these extracts, as well as the aqueous extract, were found to be less active antiscorbutically than would have been expected if their capacity for reducing indophenol was due solely to ascorbic acid. There was present in these extracts at least one other reducing substance which, though it reduced indophenol more slowly than ascorbic acid in the last stages of the titration, reduced this indicator fairly quickly at the beginning of the titration, particularly when its concentration was relatively high. It is evident, therefore, that the presence of such substances would seriously vitiate the end-point of an ascorbic acid estimation by indophenol.

It is highly probable that the other extracts (trichloroacetic acid and sulphuric acid), which possessed reducing capacities of a similar order and which showed similar chemical properties to the above, would also be less potent antiscorbutically than the peas from which they were obtained.

With regard to the second problem of this investigation, it seems certain from the evidence obtained that the activity of the peas is due entirely to the reduced form of the vitamin. It was seen that treatment with hydrogen sulphide did not increase the indophenol-reducing capacity of the extracts as would have been the case if the active oxidised form of the vitamin had been present. Further, the presence of the latter would have imparted to them a higher antiscorbutic activity than would have been anticipated from the indophenol titrations instead of a lower.

The lesser activity of the extracts than that of the peas from which they were prepared can best be explained on the grounds that it is not possible, by

the methods used, to extract the vitamin completely. This, incidentally, shows the ease with which misleading results may accrue from testing extracts instead of tissues.

If it should be that a part of the vitamin is bound to "Hemmungstoffe," as Euler suggests, it would seem most probable that such a complex would be insoluble or otherwise inextractable.

The stabilising influence of phosphate on the vitamin, which is at present inexplicable, has already been indicated by the work of Euler, Myrbäck and Larsson [1933].

SUMMARY.

1. A number of extracts have been prepared from peas germinated for 3 days and both their indophenol-reducing capacities and their antiscorbutic activities have been determined.

2. The extracts with even the highest indophenol-reducing capacity were found to possess only half the antiscorbutic activity of the germinated peas from which they were obtained.

3. Aqueous, phosphate and cyanide-phosphate extracts were found to be less active antiscorbutically than would have been expected if their indophenol-reducing capacity had been entirely due to ascorbic acid.

4. All extracts from the germinated peas contain at least one substance other than ascorbic acid which reduces indophenol.

5. No evidence was obtained which shows that the active oxidised form of the vitamin is present in the early stages of germination.

6. The ungerminated peas, which showed no antiscorbutic activity when tested in quantities of 2.5 g., gave extracts which also reduced indophenol, though at a rather slower rate than ascorbic acid.

My thanks are due to Dr S. S. Zilva for help and criticism and to the Medical Research Council for a whole time grant.

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CCLXV. THE BODY FATS OF THE PIG.

III. THE INFLUENCE OF BODY TEMPERATURE ON THE COMPOSITION OF DEPOT FATS.

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(Received November 7th, 1933.)

HENRIQUES AND HANSEN [1901] compared the setting-points and iodine values of fats from the leaf and from different layers of the back fat of a pig, which had been fed on barley. From the results (summarised below) they concluded that the determining factor in the relative hardness or softness of the fats was the temperature of the site in which the fat had been laid down in the animal:

	Solidifying point ° C.	Iodine value		Body tem- peratures ° C.
Outer back fat				
{outermost	—	60.0		
{inner layer	26.4	57.1		
Inner back fat				
{outer layer	28.0	51.8		
{innermost	27.7	50.6		
Perinephric fat	29.6	47.7		
			Back tissue { 1 cm. deep	33.7
			2 "	34.8
			3 "	37.0
			4 "	39.0
			Rectum	39.9

They obtained further support for this hypothesis by maintaining three pigs from the same litter for two months at different (but in each case approximately constant) temperatures—one at 30–35°, one at 0° and one at 0° but covered with a sheepskin coat: the iodine values of the outermost layers of the back fats from these animals, at the end of the period, were respectively 69.4, 72.3 and 67.0.

In previous parts of this series [Bhattacharya and Hilditch, 1931; Banks and Hilditch, 1932], in the course of detailed analyses of fats from a number of pigs fed on various diets, the differences in the amounts of component acids which determine the general hardness or softness of the fats as a whole were defined. *Inter alia*, and in agreement with Henriques and Hansen, it was observed in all cases that the average composition of the outer layer of back fat (between the skin and the "streak," or thin layer of connective tissue) differed from that of the fat on the inner side of the streak. This difference consisted in the presence of somewhat less stearic and palmitic acids, and of correspondingly greater proportions of oleic acid, in the fat of the outer as compared with the inner layer; the proportions of linoleic and of myristic acid were almost the same in each layer.

The primary object of the experiments recorded in the present paper was to ascertain whether the respective layers of fat on either side of the "streak" are homogeneous, or whether there is a progressive alteration as the skin is approached; in other words, to extend the earlier work of Henriques and Hansen by obtaining more definite information concerning the component fatty acids in successive lateral sections of the back fat of a pig by means of the modern ester fractionation procedure. For this purpose, the central portion of the

whole of the back fatty tissue from a very fat sow was divided into five layers of approximately equal thickness (two from the "outer" portion between the skin and "streak," and three from the "inner" portion beneath the "streak").

The sow had been fed exclusively on a diet of maize meal, thirds and whey, and care was taken to select an animal which had not received any fish meal in order to exclude any possible entrance of marine oil acids from the latter into the body fat. The unusually high linoleic acid content and appreciable, if small, amounts of highly unsaturated acids of the C_{20} and C_{22} series observed by Banks and Hilditch [1932] in the body fats from a sow, whose food had contained 7 % of fish meal, had been attributed to the presence of the latter in the diet. In their proportions of linoleic and highly unsaturated C_{20} and C_{22} acids, however, the fats from the present animal were found to be remarkably similar to those of the previous case, and, as discussed later, it would now seem that, in these instances, the cause must be sought elsewhere than in the fish meal components of the feed.

The fat was extracted from the tissues by boiling them twice with acetone, after which the residual tissue was thoroughly pressed and finally washed three times with hot acetone. The last traces of solvent were removed from the fats by heating under vacuum at 100° .

Table I.

	Fat in tissue %	Extracted fat			Mixed acids setting-point $^{\circ}$ C.
		Saponification equivalent	Iodine value	M.P. (open tube) $^{\circ}$ C.	
Outer layer					
outside ("Outer I")	66	287.1	70.4	30.3	37.7
inside ("Outer II")	72	286.9	67.4	33.3	39.4
Inner layer					
outermost ("Inner I")	91	286.2	62.9	38.0	40.8
middle ("Inner II")	92	286.3	63.0	38.3	41.0
innermost ("Inner III")	88	286.5	62.8	38.3	40.9

The component acids present in each fat were determined by the same procedure (separation by means of the lead salts into "solid" and "liquid" acids, followed by fractional distillation under vacuum of the methyl esters of each of these groups of acids) as that followed in the previous study of sow body fat [Banks and Hilditch, 1932]. As in the latter case, the higher and the residual fractions from the methyl esters of the "liquid" acids possessed saponification equivalents and iodine values which indicated, in each instance, the presence in small amounts of highly unsaturated acids of the C_{20} and C_{22} series; their proportions were approximately calculated by the same method as that described in the paper cited.

Since it has recently been shown that palmitoleic, or a similar, acid occurs in small quantities in some body fats, for instance, in that of the rat [Banks *et al.* 1933], a portion (91.6 g.) of the methyl esters of the "liquid" acids from fat "Inner III" of the present series was distilled separately from that employed in the main analysis. About one-third of the whole (31.0 g.) was collected as a first fraction; this quantity was sufficient to include practically the whole of any unsaturated esters of acids lower in the series than oleic acid. This fraction was oxidised in acetone solution with potassium permanganate until practically all unsaturated material had disappeared and yielded 5.1 g. of almost saturated esters (iodine value 0.8, saponification equivalent 263.0). Since the original fraction oxidised had an iodine value of 89.8 and a saponification equivalent of

286.0, it follows that the unsaturated esters present therein must have had an iodine value of 107.3 and a mean equivalent of 292; the purest fraction of unsaturated C_{18} esters subsequently isolated at a later part of the original fractionation had iodine value 107.0, saponification equivalent 294.0. From this experiment it appears very unlikely that unsaturated acids of lower molecular weight than oleic acid occur in the body fat of the pig; if present, they cannot form much more than about 1 % of the component fatty acids.

Table II. *Summarised data for component fatty acids of the sow back fats.*

Acid	Solid acids S	Liquid acids L	Total	Fatty acids (excluding unsaponifiable matter)	
				% (wt.)	% (mols.)
Outer I (outer layer, outside). (203.0 g.*)					
	(33.7 %)	(66.3 %)			
Myristic	0.27	2.30	2.57	2.6	3.1
Palmitic	22.61	1.19	23.80	23.8	25.6
Stearic	10.12	—	10.12	10.1	9.8
Oleic	0.70	45.53	46.23	46.3	45.0
Linoleic	—	15.13	15.13	15.2	14.8
C ₂₀₋₂₂ unsaturated	—	1.96	1.96	2.0	1.7
Unsaponifiable	—	0.19	0.19	—	—
Outer II (outer layer, inside). (201.7 g.)					
	(37.0 %)	(63.0 %)			
Myristic	0.14	2.66	2.80	2.8	3.4
Palmitic	22.84	0.58	23.42	23.5	25.1
Stearic	12.98	—	12.98	13.0	12.6
Oleic	1.03	41.87	42.90	43.0	41.8
Linoleic	—	15.59	15.59	15.6	15.3
C ₂₀₋₂₂ unsaturated	—	2.09	2.09	2.1	1.8
Unsaponifiable	0.01	0.21	0.22	—	—
Inner I (inner layer, outermost). (203.7 g.)					
	(39.6 %)	(60.4 %)			
Myristic	0.39	2.53	2.92	2.9	3.5
Palmitic	24.23	0.57	24.80	24.9	26.6
Stearic	14.51	—	14.51	14.5	14.0
Oleic	0.47	42.19	42.66	42.7	41.4
Linoleic	—	13.85	13.85	13.9	13.6
C ₂₀₋₂₂ unsaturated	—	1.06	1.06	1.1	0.9
Unsaponifiable	—	0.20	0.20	—	—
Inner II (inner layer, middle). (200.8 g.)					
	(39.7 %)	(60.3 %)			
Myristic	0.20	2.57	2.77	2.8	3.3
Palmitic	24.44	0.97	25.41	25.5	27.3
Stearic	14.49	—	14.49	14.5	14.0
Oleic	0.57	40.70	41.27	41.3	40.1
Linoleic	—	14.46	14.46	14.5	14.2
C ₂₀₋₂₂ unsaturated	—	1.44	1.44	1.4	1.2
Unsaponifiable	—	0.16	0.16	—	—
Inner III (inner layer, innermost). (310.0 g.)					
	(39.3 %)	(60.7 %)			
Myristic	0.08	2.92	3.00	3.0	3.6
Palmitic	23.78	0.72	24.50	24.6	26.2
Stearic	14.53	—	14.53	14.5	14.0
Oleic	0.90	41.84	42.74	42.8	41.6
Linoleic	—	13.66	13.66	13.7	13.4
C ₂₀₋₂₂ unsaturated	—	1.40	1.40	1.4	1.2
Unsaponifiable	0.01	0.16	0.17	—	—

* Weight of mixed fatty acids employed in the analysis.

Apart from these points, the fractionation analyses followed our usual practice, and it is perhaps sufficient (owing to considerations of space) to summarise the results (Table II) in the form adopted in several recent communications from this laboratory.

The relationships between the various component acids of this series of fats, compared on the basis of molar percentages, will be seen more clearly from the summary in Table III.

Table III.

(i) Molar distribution of individual acids.						
Fat	Myristic	Palmitic	Stearic	Oleic	Linoleic	C ₂₀₋₂₂ unsaturated
Outer I	3.1	25.6	9.8	45.0	14.8	1.7
„ II	3.4	25.1	12.6	41.8	15.3	1.8
Inner I	3.5	26.6	14.0	41.4	13.6	0.9
„ II	3.3	27.2	14.0	40.1	14.2	1.2
„ III	3.6	26.2	14.0	41.6	13.4	1.2

(ii) Molar percentages of the various groups of acids.				
	Total saturated acids	Total C ₁₈ acids	Stearic acid	Molar ratio of saturated to unsaturated acids
Outer I	38.5	69.6	9.8	0.63
„ II	41.1	69.7	12.6	0.70
Inner I	44.1	69.0	14.0	0.79
„ II	44.5	68.3	14.0	0.80
„ III	43.8	69.0	14.0	0.78

DISCUSSION.

Before comparing the differences between the successive layers of fat from the back of this animal, we would point out that all the five fats share in common an unusually high content of linoleic acid and also a proportion of highly unsaturated C₂₀₋₂₂ acids which, though small, is definitely higher than the amount (up to 0.4 %) shown by Brown and Deck [1930] to be characteristic for normal lards. Further, so far as these particular acids are concerned, all the present fats display a great similarity to those previously studied from a sow whose diet had contained about 7 % of fish meal.

In the present instance the diet cannot well account for these peculiarities. It is unlikely that fat present in the maize meal is a contributory factor since, although this fat is of a fairly unsaturated type, it is no more so than that of barley or oats, which have been employed by other workers and which in their experience have not given rise to this type of body fat in pigs. Moreover, the sow fats now in question show the normal proportion of palmitic and myristic acids, whereas the work of Ellis *et al.* [1931] showed, as the result of feeding cottonseed oil (a fat similar in linoleic acid content to maize oil) to pigs, that the proportion of palmitic acid in the hog back fats fell markedly as that of linoleic acid rose (with increasing proportions of cottonseed oil in the diet). In any case, of course, the appreciable amounts now observed of unsaturated C₂₀ and C₂₂ acids cannot be associated with any part of the diet.

We are accordingly inclined to the view that the high linoleic acid and appreciable unsaturated C₂₀₋₂₂ acid contents of the sow fats are due to some cause other than the diet. The most obvious difference in the two categories is, as a matter of fact, in the ages of the animals from which the fats were taken. In the studies of Ellis *et al.* [1926; 1930; 1931] on the body fats of hogs fed on rations low in fat, and also in those of Bhattacharya and Hilditch [1931], and in

other cases in which a low content of linoleic acid (from 1 % to not more than about 8 %) has been observed, it is noticeable that the fats were taken from young animals not more than about seven or eight months old. The two sows, whose fats have been examined and found to contain about 13–15 % of linoleic acid and 1–2 % of highly unsaturated acids of the C_{20} and C_{22} series, were probably several years old when slaughtered. It thus seems very possible that these general differences in body-fat composition may be connected with the age of the animal rather than with its diet.

Turning now to the component fatty acids of the different layers of back fat from the same animal (Table III), it must be said that the composition of each of the three "inner" layers is identical, or, at least, any variation is within the limits of experimental error of the ester-fractionation method. The part of the "outer" layer next to the "inner" layer (but divided from it by the "streak") is also very similar to the "inner" layers, but here the stearic acid content is definitely lower. The outermost layer of all differs most in composition from the rest, for in this case the stearic acid content is about 4 units % lower, and the oleic acid correspondingly higher, than in the "inner" layer fats. Both sections of the "outer" layer of fat also contain slightly less palmitic acid than the "inner" layers, but this difference is not so marked as in the case of the stearic acid figures.

In Part II of this series of papers [Banks and Hilditch, 1932] it was pointed out that, in the depot fats of pigs and cattle, the molar content of C_{18} acids is always in the neighbourhood of 70 %, rising to about 73 % with very unsaturated fats and falling to about 65 % in the more saturated fats, and that the actual degree of relative saturation is controlled, almost wholly, by the relative proportions of stearic and oleic acids (linoleic acid being more or less constant in proportion for different fats from different parts of the body of the same animal). This feature is well illustrated by the present group of fats, in which the softer (outer) fats contain about 69.7 % of total C_{18} acids in their component acids, whilst the more saturated (inner) fats contain 69.0 % or somewhat less. The main difference responsible for the difference in consistency lies in the varying proportions of stearic (9.8–14.0 %) and oleic (45.0–*ca.* 41.5 %) acids.

The progressive increase in mean saturation and the close identity in composition of all the "inner" layers of fat are perhaps best seen by reference to the final column of Table III (ii), in which the molar ratios of saturated to unsaturated acids as a whole in each fat are given.

On the whole, the present data confirm the conclusion of Henriques and Hansen that the increase in saturation (*i.e.* in stearic acid content) of the back fat follows the increase in body temperature. The figures given by these workers for the "inner layers" of fat (iodine values 51.8 and 50.6) also indicate close similarity in composition, although the respective body temperatures quoted were somewhat different, *viz.* 37.0° and 39.0°. In our experiments, the actual amount of fat of constant composition formed at least two-thirds of the whole back fat, and increasing unsaturation (oleic acid content) was only marked in the extreme outer layers. Since, however, the total thickness of the back fat of our animal appears to have been greater than that of the pig studied by the earlier workers, we consider that this may well account for the minor differences between the two series of observations, and that our detailed analyses confirm, so far as the fat nearest the skin is concerned, the apparent connection between the fat composition and the body temperature which Henriques and Hansen pointed out.

Cuthbertson and Tompsett [1933] have recently observed that the outer and

inner layers of the panniculus adiposus abdominalis in obese human subjects possess substantially the same iodine values and suggest therefrom that an explanation of variation in fat composition based on dietary considerations is more probable than one based on temperature differences. It would appear, however, that, in the case of human subjects, the effect of clothing would minimise any temperature differences. The results obtained by Cuthbertson and Tompsett are accordingly of the nature which would be expected, in view of Henriques and Hansen's experiment with a pig wrapped in a sheepskin. More detailed examination of the fat from human adipose tissues than the mere determination of iodine values would have been of extreme interest.

We venture to add a few words upon the broader question of the supposed general dependence of fat composition in plants and in animals on the temperature at which the fat is laid down—a matter upon which there seems to be some confusion of thought at the moment. Thus, Hammond [1933] has recently said that "Fat to be of use as a source of energy in the body must be just fluid at the natural body temperature, and as a consequence the fat of cold-blooded animals (fish) is of very low melting-point, while the fat of the sheep which has a high body temperature (104° F.) is of higher melting-point than that of the bullock with a lower body temperature (101° F.)." Whilst it is clear that fats present in an animal (or plant) must be almost completely, if not wholly, liquid at the natural temperature of the organism, it does not necessarily follow that warm-blooded animals or tropical plants always produce fats of higher melting-point and more saturated character than cold-blooded animals or plants which are indigenous to cool regions.

The instances of fats of fish, sheep and bullock given in the quotation, for example, should be considered in conjunction with those of such animals as the rabbit (body temperature $103\text{--}104^{\circ}$ F.) or the hen ($104\text{--}108^{\circ}$ F.). Whilst sheep fat contains only about 40 % of unsaturated acids (mainly oleic), rabbit fat contains nearly 70 %, most of which is linoleic acid, with appreciable quantities of still less saturated acids. Again, hen fat contains about 70 % of unsaturated (oleic and linoleic) acids, in spite of the high body temperature of the bird; this fat is, indeed, almost completely liquid at room temperature. Further, the rat, with a body temperature of 100° F. (lower than that of the rabbit) contains about the same high proportion of unsaturated acids [Banks *et al.*, 1933], but these consist almost wholly of oleic acid: the more unsaturated linoleic acid, present in great quantity in rabbit fat, is almost absent from that of the rat.

It has also become usual to connect the liquid, very highly unsaturated fats of fish with their low body temperature; yet marine mammals such as the whale, dugong ($102\text{--}104^{\circ}$ F.) or porpoise ($96\text{--}98.6^{\circ}$ F.) have body temperatures of the same order as those of land animals, whereas their fats are very closely similar in composition to the fish oils, and include the same series of highly unsaturated acids.

Similarly, the occurrence of fats, solid at the normal temperature of temperate regions, in the seeds of the members of a fairly large number of tropical plant families has led to the superficial generalisation that plants of tropical origin yield fats of high melting-point and those of cooler regions fats of low melting-point. Low-melting vegetable fats are, in actual fact, common to both temperate and tropical plants, and, while it is obviously true that tropical families such as *Palmae* or *Guttiferae* yield solid seed fats with high contents of combined lauric, stearic or other saturated acids, it is equally true that many plants of exclusively tropical habitat produce seed fats of a liquid and highly unsaturated character. Familiar instances are those of *Hevea brasiliensis*.

(rubber seed oil), *Perilla ocimoides* (perilla oil), *Aleurites Fordii* (China wood oil), *Couepia grandiflora* (oiticeica oil), *Carthamus tinctorius* (safflower seed oil), *Gossypium* sp. (cotton seed oil), whilst many others might be given.

No wide generalisation can therefore safely be drawn between temperature and the composition of natural fats as a whole. Fats which are solid at the normal temperature of plants or animals are obviously incompatible with their conditions of life, but, of animals and plants which exist under relatively warm conditions, some utilise fats of a relatively saturated (solid) character, but others resemble the cold-blooded animals and temperate plants in having fats of a more unsaturated and liquid type. The chief correlation which at present seems possible is rather with their biological relationships and classification than with temperature.

SUMMARY.

The back fat of a sow was divided into five layers and the composition of the mixed fatty acids present in each layer was determined. The component acids of the three "inner" layers (on the underside of the "streak") were found to be practically identical, but the outermost layer contained slightly less palmitic acid and about 4 % less stearic acid, these being compensated by the presence of correspondingly more oleic acid. The inner part of the "outer" layer was intermediate in composition between the last-mentioned fat and the three "inner" layers, but resembled the latter more closely.

The detailed analytical figures afford general confirmation of the conclusion of Henriques and Hansen [1901], that there is a close relation between increase in saturation of the fat and increasing body-temperature. At the same time, the greater part of the back fat now examined (*i.e.* the portion beneath the "streak") was evidently completely homogeneous and made up of the same mixture of mixed glycerides containing constant proportions of the various component acids.

The animal, from which the present fat was obtained, had received a diet of maize meal, thirds and whey; but the depot fatty acids were characterised throughout by the presence of about 15 % of linoleic acid and 1-2 % of highly unsaturated acids of the C_{20} and C_{22} series. These values are probably not due to the diet of the animal but are characteristic of pigs of considerable age.

The general statement, not infrequently encountered, that warm-blooded animals and plants of tropical origin produce more solid, saturated fats than cold-blooded animals or plants from cool regions is shown to be only partially true.

We are indebted to Dr E. M. Cruickshank for very kindly collecting for us some of the information relating to the body temperatures of various animals.

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CCLXVI. THE SPECIFIC ROTATION AND STABILITY OF (2, 5)-FRUCTOSE FROM A MATHEMATICAL STUDY OF THE HYDROLYSIS OF SUCROSE BY FRUCTOSACCHARASE.

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(Received September 24th, 1933.)

ALTHOUGH the sugars and polysaccharides have been successfully investigated by purely chemical methods in recent years, certain points in the complete elucidation of their structure remain obscure. For example, methods involving oxidation, reduction and methylation afford no clue to the type of linking between sugar molecules, but by utilising enzymes of appropriate specificity it has been possible to assign a specific linking to the hexose molecules composing some of the commoner di- and tri-saccharides.

The present communication concerns the use of yeast fructosaccharase as an agent in the elucidation of the specific rotation of (2, 5)-fructose as liberated from sucrose, and of the unimolecular velocity constant of the change of (2, 5)-fructose to equilibrium fructose.

From evidence derived by methylation procedure, sucrose appears to be 1-(1, 5)-glucosido-2-(2, 5)-fructoside [Avery *et al.*, 1927; Haworth *et al.*, 1927, 1, 2]. The configuration of the fructose 2-carbon atom is an open question. That of the glucose 1-carbon atom also is regarded by Haworth in the same light, but nevertheless there are the following reasons for assigning the α -form to the glucose component.

(a) Hudson [1908] found that after an almost instantaneous hydrolysis of sucrose by invertase, the mutarotatory fall continued long after the mutarotatory effects due to the fructose portion were presumed to have ceased. (The mutarotation of normal fructose is much more rapid than that of glucose, and in the present instance, as will be shown, one reaction involved is even faster, *viz.* the change of fructofuranose to fructopyranose.) Hudson concluded that the α -mutarotation at so late a period was due to α -glucose.

(b) Sucrose is not hydrolysed by emulsin (β -glucosidase).

(c) According to Weidenhagen [1929], yeast maltase (α -glucosidase) hydrolyses sucrose as rapidly as maltose at p_H 7, the optimum for the enzyme, but hydrolyses neither sucrose nor maltose at p_H 4.7, the optimum for fructosaccharase (β -*h*-fructosidase), so that the reaction at p_H 7 was not due to the latter enzyme. If this is confirmed, it affords evidence of the presence of α -glucose in the sucrose molecule.

Hudson [1909] could observe no striking change in rotation at the outset of his very rapid hydrolysis, and he concluded that the combined rotations of the products were equal to that of the sucrose. Assuming the one component to be α -glucose, he calculated the specific rotation of the fructose component as follows.

Since 1 g. of sucrose ($[\alpha]_D = +66^\circ$) yields 0.525 g. of glucose ($[\alpha]_D = +109^\circ$) and 0.525 g. of fructose ($[\alpha]_D = x$), then $0.525(109 + x) = 66$, *i.e.* $x = +17^\circ$.

Hudson's deduction from qualitative data is not entirely satisfactory, since nothing was known of the transformation of fructofuranose to equilibrium fructose; if this change were instantaneous or nearly so, it is evident that an inability to record an initial rise in rotation would be no foundation for the above calculation. The results obtained in the present investigation from strictly kinetic data, have, however, confirmed Hudson's calculation and have shown that the change from fructofuranose to equilibrium fructose is unimolecular, $k_{17^\circ}^{(\text{mins.}^{-1})} = 0.3$, between p_H 4.6 and 6.1.

With suitable concentrations of yeast fructosaccharase and sucrose, it is possible to obtain an accurately unimolecular inversion up to the stage of some 60–70 % hydrolysis, and it is with data furnished during this period that the present investigations are concerned. The rotational change of the enzyme sucrose mixture, suitably buffered, is followed polarimetrically. To aliquot portions of the same reaction mixture is added from time to time dilute ammonia in suitable volumes to arrest hydrolysis and to accelerate mutarotation. The final rotations of these portions, after correction for the slight rotation of the enzyme preparation and the dilution by ammonia, indicate the extent of inversion at the respective times. The results of such an experiment in which inversion was carried to the end of the strictly unimolecular stage are given in Table I (Exp. 1) and shown graphically in Fig. 1, in which observed rotations are plotted against time. Curve (b) represents the rotation of the mutarotating solution, curve (c) that of the mutarotated solution after applying the necessary correc-

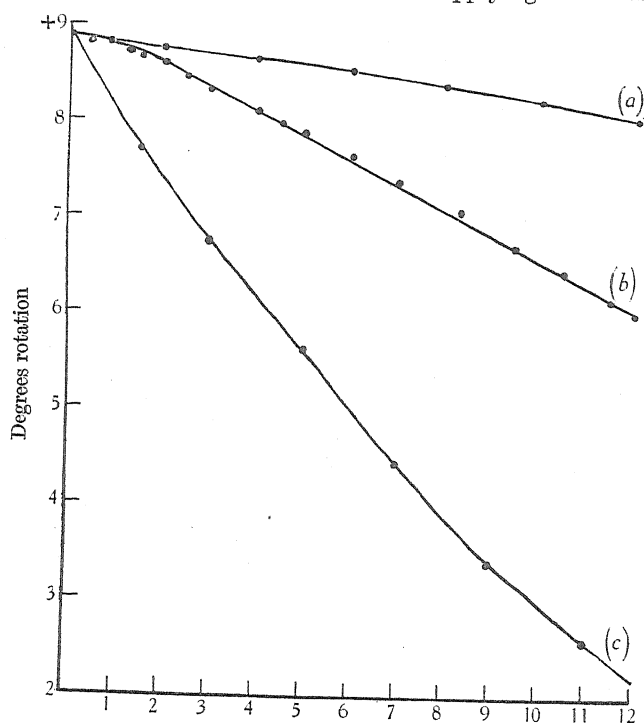


Fig. 1. Inversion of sucrose by fructosaccharase. Temperature 17° . p_H 4.6.

- (a) Rotation sucrose + mutarotating glucose.
- (b) Rotation sucrose + mutarotating glucose and fructose.
- (c) Rotation sucrose + mutarotated glucose and fructose.

100 cc. 20 % sucrose, 10 cc. *N*/10 acetate buffer, η_{H} 4-6, total volume, 150 cc. Temperature 17°. Volumes of enzyme preparation: Experiment 1, 20 cc.; Experiment 2, 30 cc. of a more active preparation.

Polarimeter readings. Corrected for rotation of enzyme										SPECIFIC ROTATION OF (2, 5)-FRUCTOSE					1959	
Mutarotating portion				Mutarotated portion		Arbitrary times mins.	Sugars present		Rotation due to	Fructose (from graph, Fig. 1)	[α] _D of fructose	Ratio L.H.S. R.H.S. (equation 5)				
Time mins.	Angular degrees	Time mins.	Angular degrees	Time mins.	Angular degrees		Sucrose g.	Glucose or fructose (g.) Experiment 1								
0	+8.88	0.0	+8.88	—	—	0.8	—	—	—	0.00	0.0	0.96				
0	8.81	1.5	7.71	0.0306	—	2.0	17.46	1.34	+7.75	+1.00	-12.3	0.97				
0	8.81	3.0	6.74	0.0293	—	4.0	15.20	2.53	+6.74	+1.88	-30.8	0.94				
1	15	8.74	—	—	—	6.0	13.30	3.53	+5.91	+2.62	-39.5	0.98				
1	30	8.67	5.0	0.0301	—	8.0	11.54	4.45	+5.12	+3.26	-44.2	1.05				
2	0	8.61	—	—	—	10.0	9.93	5.29	+4.41	+3.82	-47.9	1.07				
2	30	8.46	7.0	0.0298	—	12.0	8.51	6.04	+3.78	+4.28	-50.2	1.10				
3	0	8.32	—	—	—	Mean	—	—	—	—	—	Mean				
4	0	8.11	—	—	—	<i>k_s</i> (<i>ln</i>)	—	—	—	—	—	1.01				
4	30	7.98	9.0	0.0305	—	<i>k_s</i> (<i>ln</i>)	—	—	—	—	—					
5	0	7.89	—	—	—	(base <i>c</i>)	—	—	—	—	—					
6	0	7.64	2.0	0.0305	—											
7	0	7.37	—	—	—											
8	20	7.09	13.5	0.0315	—											
9	30	6.73	—	—	—											
10	30	6.47	—	—	—											
11	30	6.17	—	—	—											
12	30	5.92	—	—	—											
13	15	5.68	—	—	—											
14	0	5.48	—	—	—											
15	0	5.26	—	—	—											
0	+8.88	0.0	+8.88	—	—	1.0	17.93	1.10	+7.96	+0.80	0.0	1.01				
0	8.72	1.66	6.97	0.0468	—	2.0	16.08	2.07	+7.14	+1.51	-11.6	0.99				
0	8.75	2.83	5.76	0.0477	—	4.0	13.15	3.60	+5.83	+2.71	-27.5	0.97				
1	2	8.65	4.50	0.0454	—	6.0	10.53	4.98	+4.68	+3.65	-34.3	1.04				
1	40	8.53	3.30	0.0463	—	7.5	8.82	5.88	+3.91	+4.24	-37.3	1.08				
2	20	8.38	6.08	0.0463	—	Mean	—	—	—	—	—	Mean				
3	5	8.22	7.58	0.0476	—	<i>k_s</i> (<i>ln</i>)	—	—	—	—	—	1.02				
3	30	7.98	9.0	0.0481	—	No longer unimolecular	—	—	—	—	—					
4	15	7.80	10.5	0.0486	—											
5	0	7.53	—	—	—											
6	0	7.23	—	—	—											
6	30	7.04	—	—	—											
7	0	6.82	—	—	—											
8	0	6.52	—	—	—											
8	30	6.33	—	—	—											
9	0	6.18	—	—	—											
10	0	5.90	—	—	—											
11	0	5.50	—	—	—											

Mean.
1.08
1.02

tions, curve (a) that of unchanged sucrose *plus* mutarotating glucose, arrived at by calculation as indicated below. The difference between curves (a) and (b) at any time point therefore represents the rotation due to fructose at that instant. Curve (a) was arrived at from the following considerations—which apply only to the unimolecular period of the hydrolysis. Let a = initial amount of sucrose (g. in v cc.), $(a - 2q)$ = sucrose remaining after time t , q being glucose or fructose liberated in time t , uncorrected for water taken up on hydrolysis. (The necessary correction is applied later.)

$$\text{Then} \quad k_s = \frac{1}{t} \ln \frac{a}{a - 2q} \quad \dots\dots(1),$$

where k_s is the unimolecular velocity constant of hydrolysis of sucrose.

$$\text{Differentiating} \quad dq = \frac{a}{2} \cdot k_s \cdot e^{-k_s t} dt \quad \dots\dots(1 a).$$

Now consider the mutarotatory change of α -glucose to $\alpha\beta$ -glucose, which again is a unimolecular reaction. Let the unimolecular velocity constant of this change be k_G

$$k_G = \frac{1}{t} \ln \frac{P_0 - P_\infty}{P - P_\infty} = \frac{1}{t} \ln \frac{c}{P - P_\infty} \quad (\text{putting } P_0 - P_\infty = c),$$

where P_0 , P and P_∞ are the specific rotations of the glucose at times t_0 , t and t_∞ .

$$\text{From this,} \quad P = ce^{-k_G t} + P_\infty \quad \dots\dots(2).$$

Using P for specific rotations and ρ for actual rotations under observation, the rotation $d\rho$ of dq grams of α -glucose after an interval of time t , will be:

$$d\rho = \frac{dq}{v} (ce^{-k_G t} + P_\infty).$$

Substituting the value of dq from equation (1 a)

$$d\rho = \frac{a}{2v} \cdot k_s e^{-k_s t} (ce^{-k_G t} + P_\infty) dt.$$

The total rotation ρ , of Σdq grams of mutarotating α -glucose after time t will be,

$$\int d\rho = \int_0^t \frac{a}{2v} (k_s ce^{-(k_s + k_G)t} + k_s e^{-k_s t} P_\infty) dt, \\ \text{i.e.} \quad \rho = \frac{a}{2v} \left(P_\infty + \frac{k_s c}{k_s + k_G} - P_\infty e^{-k_s t} - \frac{k_s c}{k_s + k_G} \cdot e^{-(k_s + k_G)t} \right) \quad \dots\dots(3).$$

As an example, the values substituted in the case of Exp. 1 of Table I were $a = 20$, $v = 150$, $c = P_0 - P_\infty = 111.2 - 52.5 = 58.7$, $k_s = 0.07$, $k_G = 0.0115$.

(This value of k_G was taken from the data furnished by Nelson and Beegle [1919], who investigated the velocities of mutarotation of glucose and fructose at various temperatures and values of p_H .) The values of ρ so obtained were multiplied by $\frac{20}{19}$, since 19 g. of sucrose yield 20 g. of hexose. These values were added to the calculated rotations due to unchanged sucrose at the corresponding times respectively and plotted to give curve (a). The difference in reading between curves (a) and (b) at any time gives the rotation due to fructose at that time. Let this difference be F . Then the specific rotation of the fructose present, *i.e.* the mean specific rotation of Σdq , where each small increment is at a different stage of mutarotation, is $Fv/\frac{20}{19}q$.

These values are tabulated for each experiment (Exps. 1-5, Tables I-II) and plotted collectively against time in Fig. 2. These curves express the relation between specific rotation of fructose produced at known unimolecular rate and time. Extrapolation to the ordinate gives in all cases the value $+15^\circ$ to $+17^\circ$ for the specific rotation of the fructose as first liberated, *i.e.* for (2, 5)-fructose. This low positive rotation would suggest that the (2, 5)-fructose is in the β -form.

Table II.

Reaction mixtures as in Exps. 1 and 2. Temp. 17°.

Exp.	Volume of enzyme preparation	$k_s^{(mins.-1)}$ base e_s (mean value)	Arbitrary times (mins.)	$[\alpha]_D$ of fructose	Ratio $\frac{L.H.S.}{R.H.S.}$ (equation 5)
3	30 cc.	0.0926	0.9	0.0	0.94
			2.0	-12.9	0.96
			3.0	-22.75	0.96
			4.0	-28.1	0.97
			5.0	-32.9	0.99
			6.0	-34.7	1.03
			8.0	-39.9	1.08
			Mean		0.99
4	40 cc.	0.1036	1.0	0.0	0.96
			2.0	-12.6	0.97
			4.0	-28.0	0.96
			6.0	-33.70	1.04
			8.0	-38.7	1.09
			Mean		1.00
5	40 cc. of a more active preparation	0.112	1.0	—	—
			2.0	-10.5	0.97
			3.0	-20.1	1.00
			4.0	-25.8	1.00
			6.0	-32.4	1.06
			7.5	-36.6	1.09
			Mean		1.02
6	As in Exp. 5, but at p_H 6.1	0.0857	1.0	0.0	0.97
			2.0	-12.8	1.04
			3.0	-20.9	0.96
			5.0	-33.1	0.98
			7.0	-40.9	1.00
			8.5	-44.2	1.01
			Mean		0.99

Exp. 6 (Table II, but not included in the graph) was similar to the others, but the reaction was allowed to proceed without added buffer, the p_H being 6.1. Except that the value of k_s was lower, the results, including the specific rotation of the fructofuranose, were similar to those of Exps. 1-5.

The readings of the mutarotating sugars (curve (b), Fig. 1) lie, save for an initial lag, on a straight line, which on extrapolation, cuts the ordinate at a point higher than the theoretical initial reading (checked by controls) of the sucrose before commencement of the hydrolysis. However, in some 40 experiments in which the rotation of the enzyme-sucrose mixture was read 20-30 seconds after mixing, no initial rise in rotation was ever observed. Each curve (b) when plotted from the data furnished in Tables I and II exhibits an initial lag which is not exhibited by the corresponding curve (c), and is not due therefore to a tardiness in the production of glucose and fructose. The curve (b) does not pass below the curve (a) until this lag is over. At the point of separation, the specific rotation of the fructose is exactly zero, and is subsequently negative. Before this point, it must have been positive. This accounts for the lag in curve (b). If the curve (b) fell below curve (a) directly from the theoretical initial reading, it is evident that the fructose would always exhibit a negative rotation.

It will be seen that the fall in specific rotation is less rapid for a rapid hydrolysis than for a slow one. If the change from (2, 5)-fructose to equilibrium fructose is unimolecular, the velocity constant k_f can be calculated. To deduce the values of k_f at various points along each curve in Fig. 2, and to show

that k_F is not only constant for each individual curve, but the same for all the curves (*i.e.* independent of k_s), would furnish striking confirmation of the validity of the deductions by which the curves themselves were derived.

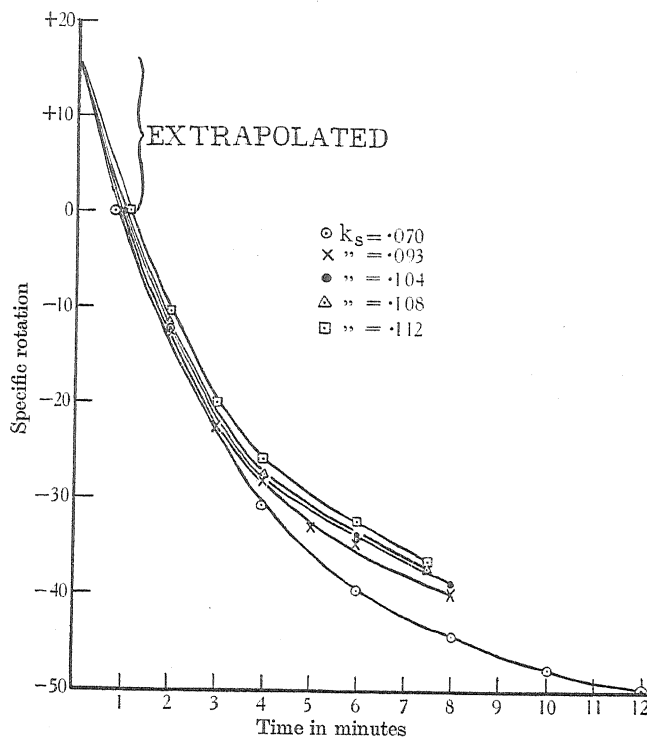


Fig. 2. The specific rotation of fructose produced unimolecularly in the hydrolysis of sucrose by fructosaccharase, at 17° , p_H 4.6.

Calculation of k_F .

By means of equation 3, the actual rotation of the glucose present could be evaluated. If the change from (2, 5)-fructose to equilibrium fructose is unimolecular, we have a reaction comparable in its manifestation with the mutarotation of the glucose. By substituting fructose constants in equation 3, and multiplying each side by $v/\frac{20}{19}q$ since we are now dealing with specific rotation, an equation is obtained which represents the course of the curves in Fig. 2,

$$\rho \cdot \frac{v}{19q} = \frac{av}{2 \cdot 20 \cdot vq} \left(P_\infty + \frac{k_sc}{k_s + k_F} - P_\infty e^{-k_st} - \frac{k_sc}{k_s + k_F} \cdot e^{-(k_s + k_F)t} \right),$$

$$\text{i.e.} \quad P = \frac{19}{40} \cdot \frac{a}{q} \left(P_\infty + \frac{k_sc}{k_s + k_F} - P_\infty e^{-k_st} - \frac{k_sc}{k_s + k_F} \cdot e^{-(k_s + k_F)t} \right) \quad \dots (4).$$

This equation has no formal solution. By rearranging,

$$e^{-(k_s + k_F)t} = 1 + \left[P_\infty - P_\infty e^{-k_st} - P \cdot \frac{40q}{19a} \right] \left[\frac{k_s + k_F}{k_sc} \right],$$

and equating y with each side of this equation and solving graphically, the value of k_F can be found. The values substituted were, P_∞ at $17^\circ = -92.3^\circ$, $P_0 = +17^\circ$, $c = +17 - (-92.3) = +109.3^\circ$, and P at any arbitrary time t , was read from

Fig. 2. Graphical solution was effected in this way for one or two values of t in each curve of Fig. 2. It was found to vary only between the values 0.28 and 0.35. It was now necessary to see whether the substitution of the mean value of k_F , viz. 0.31, in equation 4 at every arbitrary time point of the curves of Fig. 2 would give an accurate identity in all cases.

Rewriting equation 4 as

$$\underbrace{\frac{40 P q}{19 a} + P_{\infty} e^{-k_s t} - P_{\infty}}_{\text{L.H.S.}} = \underbrace{\frac{k_s c}{k_s + k_F} (1 - e^{-(k_s + k_F) t})}_{\text{R.H.S.}} \quad \text{.....(5),}$$

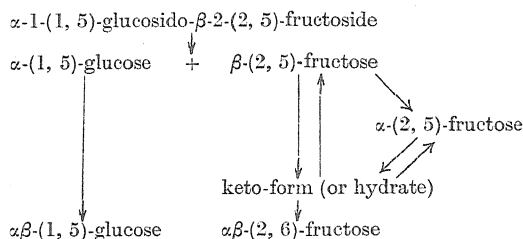
and substituting $k_F = 0.31$, and finding the ratio L.H.S./R.H.S. we obtain an idea of the extent of variation from the unimolecular ideal. It is obvious from the values of this ratio given in Tables I and II that there is only variation within reasonable limits and therefore that the assumption that the fructofuranose transformation is unimolecular is correct.

Between p_H 4.6 and 6.1, $k_F = 0.31$. The change of fructofuranose to equilibrium fructose is therefore by no means instantaneous. It is 27 times as fast as the mutarotation of α - or β -glucose under the same conditions, and 3 times as fast as that of β -(2, 6)-fructose.

Assuming, as seems most probable, that the liberated fructofuranose is in the β -form, its subsequent transformation may conceivably proceed in either or both of two directions, (1) to α -(2, 5)-fructose, (2) to (2, 6)-fructose. The latter transformation, which must involve the opening of the ring and temporary formation of the keto-form (or a hydrate of this) would be relatively rapid owing to the instability of β -(2, 5)-fructose. The former change, even if proceeding independently, would not affect the latter since, presumably, both (2, 5) forms would tend to pass through the keto-form to (2, 6)-fructose. Finally the keto-form would yield α - and β -(2, 6)-fructose directly in normal equilibrium proportions so that the relatively slow reaction velocity of mutarotation of (2, 6)-fructose would not limit the rate of change of fructofuranose to equilibrium.

There are certain reasons, into which it is not necessary to go, for believing that equilibrium fructose contains a small proportion of one or both of the (2, 5)-forms. If this is so, the change from β -(2, 5)-fructose through the keto-form to $\alpha\beta$ -(2, 6)-fructose would cease at an equilibrium point, with a relatively small residual quantity of (2, 5)-fructose which would finish presumably as $\alpha\beta$ -(2, 5)-fructose, the β -(2, 5)-form having changed to α -(2, 5)-form either through the keto-form or some other intermediate form.

The hydrolysis of sucrose may then be represented thus:



EXPERIMENTAL.

The sucrose employed was pure; $[\alpha]_D + 66.5^\circ$. The fructosaccharase was prepared from brewer's top yeast by the method of Nelson and Born [1914]. It was a clear colourless liquid, freed from inorganic salts by dialysis, rotation

+1.24° (1 dm.), contained 1.56 g. of solid matter per 100 cc. and possessed no maltase activity. It was stored at -5° with no loss in activity.

Preliminary experiments showed that:

(1) the fructosaccharase preparation had no influence on the rotation of fructose;

(2) ammonia in the concentration employed in the main experiments, completely arrested hydrolysis and had no effect on the rotation of the enzyme preparation or of sucrose, glucose or fructose;

(3) the fructosaccharase *plus* ammonia, in the same concentrations as used in the main experiments, had no influence on the rotation of sucrose.

100 cc. of 20 % sucrose (*i.e.* 107.7 g.) were mixed with 10 cc. of *N*/10 acetate buffer (p_H 4.6) and allowed to attain room temperature (17°) by standing in a bath of water for 2 hours. The enzyme preparation was similarly brought to room temperature. At time zero, 40 cc. of the enzyme solution (the exact weight was observed and the volume calculated) were added rapidly from a cylinder, and thorough mixing was achieved after 5 seconds. A polarimeter-tube with water jacket at room temperature was quickly filled and the rotation taken 20–25 seconds after mixing and at subsequent intervals. Meanwhile 15 cc. portions were withdrawn from the main portion of the reaction mixture and run into a series of small flasks contained in a water-bath at room temperature. To these at intervals 1 cc. of *N* ammonia was added with shaking, the exact time being noted after 0.5 cc. had been run in. The final rotations of the mixtures were read in a 2 dm. tube at 17° about 30 minutes later.

The success of the experiment depends entirely on temperature control, that of the main reaction mixture must not differ from that of the portion in the polarimeter-tube by more than 0.1–0.2°. The jacket of the tube was fed with water circulating through the bath and all pipettes were fitted with rubber shields.

It will be seen that the individual experiments differ only in regard to the amount of enzyme used. It was found to be of no advantage to use less than 20 cc. of enzyme (diluted to 40 cc.) ($k_s=0.07$) or more than 40 cc. ($k_s=0.11$) since in the latter case the hydrolysis was too fast to allow of accurate polarimetry.

SUMMARY.

1. The specific rotation (17°, p_H 4.6–6.1) of (2, 5)-fructose is found to be between +17° and +15°.
2. The change of fructofuranose to equilibrated fructose is shown to conform to a unimolecular law. The unimolecular velocity constant $k_{17}^{(mins.^{-1})}=0.3$.

We desire to thank Mr R. H. Roberts for assistance in the experimental work. One of us (K. B.) is indebted to the Department of Scientific and Industrial Research for a grant.

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CCLXVII. THE OXIDATION OF FRUCTOSE BY HYPOIODITE.

BY KENNETH BAILEY AND REGINALD HAYDN HOPKINS.

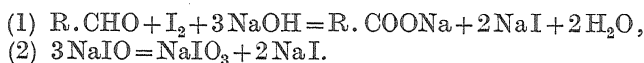
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(Received September 24th, 1933.)

THE oxidation of fructose by hypiodite, as employed in the method of Willstätter and Schudel [1918] for the estimation of aldoses, is known to be small in amount, but very variable results as to the iodine equivalent of fructose have been reported from time to time. Whereas over a fairly wide range of conditions glucose can be oxidised to the equivalent of 1.41 g. of iodine per g. of glucose, fructose has given various iodine figures under varying conditions, *e.g.* 0.1028 g. [Judd, 1920], 0.10 g. [Baker and Hulton, 1920], 0.0065–0.020 g. [Hinton and Macara, 1924], 0.018 g. at 1° [Archbold and Widdowson, 1931].

In the present communication are reported the results of an investigation of the oxidation of fructose by hypiodite under conditions similar to those used in the original Willstätter and Schudel method. The investigation has included the following: (1) effect of temperature; (2) effect of excess alkali; (3) effect of rate of addition of alkali; (4) effect of concentration of fructose; (5) control experiments; (6) identification of products of oxidation.

These investigations have shown that, if constant results are to be obtained with fructose, the most important condition to be standardised is that of the rate of addition of the alkali. Willstätter and Schudel stated that in the case of glucose the sodium hydroxide must be added slowly to the sugar-iodine solution, and it is well known that if the addition is made instantaneously, quantitative oxidation of glucose is not secured. Goebel [1927, 1] explained this on a stoichiometric basis as follows. Two reactions commence with the addition of alkali and the formation of hypiodite.



"A sufficiently high concentration of hypiodite is possibly not attained when alkali is added immediately to a glucose-iodine solution for the reason that reactions (1) and (2) start out simultaneously. Under these conditions reaction (1), though it has a greater *initial* speed than reaction (2), will be overtaken by the second as the two near completion with the result that at this point the concentration of hypiodite falls below the necessary optimum, and both reactions end with the hypiodite completely converted to iodate and iodide and the glucose in an incomplete state of oxidation. If, on the other hand, the alkali be added over an interval of 2 minutes, the major part of the hypiodite will enter into the first reaction as it is formed, . . . the concentration of hypiodite in the solution would be kept low, a condition unfavourable for the reaction hypiodite→iodate."

Goebel found that, using 0.09 g. glucose in 10 cc., 20 cc. of *N*/10 iodine, 45 cc. of *N*/10 NaOH, temp. 23°, two minutes should be taken to add the sodium hydroxide, in which case quantitative oxidation of the glucose was attained

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after 10 to 20 minutes' standing. If the alkali were added very rapidly, quantitative results were never attained, however long the subsequent standing.

The results obtained on the effect of rate of addition of alkali make it clear that the oxidation of fructose is not due to a Lobry de Bruyn-van Ekenstein transformation into oxidisable aldoses. At the same time the relatively slow oxidation of fructose by hypiodite as compared with that of aldoses renders it probable that only one component, and that a small one, of the fructose solution is actually oxidised.

EXPERIMENTAL.

The fructose employed was B.D.H. dextrose-free fructose recrystallised by Harding's method [1922]; $[\alpha]_D^{16.7} - 92.1^\circ$ ($c=5$). Calculated from Honig and Jesser's formula, $[\alpha]_D^{16.7} - 92.1^\circ$. The glucose was B.D.H., A.R., recrystallised from dilute alcohol (the first crop of crystals was discarded); $[\alpha]_D^{20} + 52.6^\circ$. Both sugars were dried *in vacuo* over P_2O_5 at 72° . All pipettes and graduated flasks were calibrated, and a standard drainage time of 30 seconds was adopted with the former. The thermostat was electrically controlled and temperature variations did not exceed $\pm 0.1^\circ$. The thiosulphate was standardised every 3 days by liberating a known amount of iodine from KI by acidified standard potassium dichromate solution.

(1) Effect of temperature.

A volume of solution containing 0.5 g. of fructose was pipetted into each of a series of 250 cc. conical flasks at room temperature and allowed to attain the requisite temperature in a thermostat. 20 cc. of $N/10$ iodine at the latter temperature were added. (In the series at 35° iodine was pipetted at room temperature, the flask closed, and the mixture allowed to attain the temperature of the thermostat before addition of the alkali. Iodine loss is rapid at 35° .) Lastly 5 cc. of $N/2$ NaOH were added over a period of exactly 20 seconds with rapid shaking. The flasks were closed, and after the appropriate time interval acidification was effected with 10 cc. of N H_2SO_4 and the liberated iodine titrated against $N/20$ thiosulphate using the Hagedorn and Jensen starch indicator. An appropriate iodine control was performed for each experiment.

Two series of experiments were performed, Series A using 10 cc. of sugar solution and a total volume of 35 cc., and Series B using 10 cc. of sugar solution + 40 cc. water, total volume 75 cc. The control experiments of Section (5) show that at room temperature the conditions of oxidation in Series A and B give the theoretical iodine value for glucose.

The oxidations were allowed to proceed at 1, 15, 25 and 35° . The extents of oxidation expressed in cc. $N/20$ iodine at various times and temperatures are given in Table I.

From these results it appears that, under the conditions adopted, the maximum oxidation was soon reached except at 1° , in which case 10 hours were required. In the last case some hypiodite was still functioning after 8 hours. In control experiments with 0.08 g. of glucose in place of the fructose, oxidation of the sugar at 1° was still proceeding after 8 hours. As will be shown (Section (3)) the extent of oxidation of fructose is very susceptible to the rate of addition of the alkali, but under the conditions of the above experiment, *viz.* a 20 second period for the addition of the alkali, the minimum oxidation of fructose which is accompanied by theoretical oxidation of glucose is effected at 15° in 20 minutes.

Table I.

Temperature ° C.	Series A		Series B	
	Time (hours)	Oxidation (cc. N/20 iodine)	Time (hours)	Oxidation (cc. N/20 iodine)
1	1.0	1.06	1.0	0.86
	3.0	2.29	3.0	1.81
	5.0	3.15	5.15	2.60
	8.0	3.75	8.0	3.07
	9.0	3.86	8.5	3.17
	10.0	4.13	10.0	3.48
	22.0	3.96	22.0	3.30
15	0.33	2.09	0.33	1.69
	0.75	3.33	0.75	2.74
	1.5	3.81	1.5	3.20
	3.6	3.73	3.5	3.20
	5.0	3.66	5.5	3.20
	9.0	3.71	9.0	3.25
	10.0	3.83	10.0	3.31
26	0.33	3.62	0.33	3.50
	1.0	3.94	1.0	3.40
	3.0	3.99	3.0	3.52
	5.0	3.99	5.0	3.52
	6.0	4.01	8.0	3.59
	7.0	4.09	10.0	3.67
	10.0	4.08		
35.5	0.33	4.16	0.33	3.94
	1.0	4.37	1.0	4.09
	3.0	4.57	2.0	4.09
	5.0	4.60	4.0	4.14
	8.5	4.77	6.0	4.11
	10.0	4.94	10.0	4.22

The final oxidation attained in both series was minimum at 15°. The extent of oxidation depends on

- (a) concentration and persistence of hypoiodite;
- (b) rate of reaction between hypoiodite and oxidisable sugar;
- (c) concentration of the oxidisable component of the fructose.

A rise in temperature favours (b), is unfavourable to (a), and is very possibly favourable to (c). Disregarding the effect of temperature on (c), effects (a) and (b) combine to produce minimum oxidation at 15°. That (a) is very favourable at 1° is shown by the continuance of oxidation after 8 hours at that temperature.

The rates of oxidation at 1° and 15° may be approximately arrived at from the first time readings (and inspection of the results in graphical form). From such observations the rates of oxidation in both series (A and B) are seen to be at least 6 times as great at 15° as at 1°. From such a result it would seem probable that factor (c) is also effective, and that there is a greater concentration of the oxidisable component at 15° than at 1°.

(2) *Effect of excess of alkali.*

In the previous experiments the oxidising system contained a small excess of alkali over that required to form hypoiodite. Series A was repeated using twice as much alkali, 10 cc. of N/2 NaOH (and equivalent increase of H₂SO₄), the total volume being thus 40 cc. The results are given in Table II.

The chief features of the effect of excess of alkali are:

- (a) a more rapid disappearance of hypoiodite at 1°, and in consequence, less total oxidation;

Table II.

1°		17°		26°		35°	
Time (hours)	Oxidation (cc. N/20 iodine)	Time (hours)	Oxidation (cc. N/20 iodine)	Time (hours)	Oxidation (cc. N/20 iodine)	Time (hours)	Oxidation (cc. N/20 iodine)
1.0	1.72	1.0	3.36	0.5	5.17	0.5	7.90
3.0	1.87	3.0	3.75	2.0	5.60	2.0	9.25
6.0	1.97	5.0	4.00	3.0	6.19	4.5	11.10
8.0	2.02	7.0	4.00	5.25	6.60	6.0	12.00
10.0	1.97	10.0	4.15	8.75	7.40	8.0	13.20
				10.00	7.97	10.0	14.75

(b) progressive oxidation at 17, 26 and 35° long after oxidation in the corresponding members of series A (Table I) had ceased and hypoiodite is presumed to have disappeared.

Excess alkali has two effects, that of accelerating the formation of iodate from hypoiodite, and that of modifying the fructose rendering it susceptible to oxidation by iodate. Low results in the determination of glucose by iodimetric titration in cases where too large an excess of alkali has been added have probably been due to the first of these causes rather than the Lobry de Bruyn transformation to which they have been ascribed.

(3) *Effect of rate of addition of alkali.*

The experimental procedure adopted for Series A and B (Table I) was repeated at room temperature only, with the difference that the 5 cc. of N/2 NaOH were added in 0.5 cc. portions at intervals of 30 or 60 minutes. Certain flasks were withdrawn at certain stages and the titration completed. The results are given in Table III.

Table III. *Effect of rate of addition of alkali.*

0.5 g. fructose, 20 cc. N/10 iodine, N/2 NaOH 0.5 cc. at a time, total volume (without NaOH), Series A 30 cc., Series B 70 cc. Temp. 16°.

Rate of addition of NaOH	Duration of reaction from first NaOH addition (hours)	Number of additions of NaOH	Series A. Oxidation (cc. N/20 iodine)	Series B. Oxidation (cc. N/20 iodine)
0.5 cc. per half hour	1.0	2	2.18	1.51
	3.0	6	7.97	5.50
	4.0	8	12.22	8.60
	5.0	10	16.76	12.24
	6.0	10	16.96	—
	8.0	10	—	12.23
	10.0	10	16.90	12.34
			(=15.3% oxidation)	(=11% oxidation)
0.5 cc. per hour	5.0	5	10.15	7.39
	10.0	10	20.20	15.70
	24.0	10	20.20	15.90
			(=18.2% oxidation)	(=14.3% oxidation)

Before discussing the results of this experiment, the following control experiments should be considered. Series A and B were repeated at 16°. Firstly, 0.5 cc. of N/2 NaOH was added to the fructose-iodine mixture, and, at definite intervals of time, acidification was effected with 1 cc. of N H₂SO₄.

Table IV.

Time (minutes)	Oxidation (cc. <i>N</i> /20 iodine)	
	Series A	Series B
20	0.90	0.90
30	1.01	0.91
40	1.16	0.96
60	1.15	1.10

Oxidation appears to have ceased within 30 minutes.

Secondly, to the fructose-iodine mixture, 0.5 cc. of *N*/2 NaOH was added every half hour until 3.5 cc. had been added. After further time intervals (as stated in Table V) acidification was effected with 5 cc. of *N* H₂SO₄.

Table V.

Time (minutes)	Oxidation (cc. <i>N</i> /20 iodine)	
	Series A	Series B
20	9.05	6.20
30	9.60	6.82
45	9.60	6.70
60	(9.80)	6.70

Here again oxidation had ceased entirely within 30 minutes.

It is evident from Table III that an abnormally high oxidation can be effected if the alkali is added slowly enough, and from Tables IV and V that this is not due to a very slow decomposition of hypoiodite. The best explanation seems to be that one component only of the fructose solution undergoes oxidation and that:

(a) each small increment of hypoiodite produces an oxidation comparable with that obtained with excess of hypoiodite;

(b) in neutral medium the removal of one component by oxidation induces the "inactive" components to establish a new equilibrium thus restoring the oxidisable component.

In this case the amount of oxidation should increase as the rate of addition of alkali decreases, which is the case (Tables I, III, VI). Comparing Tables I and III (Series A) 5 cc. of NaOH added in 20 seconds produced oxidation equivalent to 3.7 cc. of *N*/20 iodine after 5 hours, whereas 5 cc. of NaOH added in 0.5 cc. half hourly increments produced 16.76 cc. oxidation after 5 hours in all, and 5 cc., added in hourly increments, 20.20 cc. Further, 3.0 cc. of alkali after 3 hours (Tables III, Series A) had produced 7.97 cc. oxidation, whereas 5 cc. added in 20 seconds (Tables I, Series A) had produced only 3.7 cc. oxidation in 5 hours. If the whole of the fructose were susceptible to direct oxidation, then, since the quantity used was many times the molecular equivalent of the 20 cc. of *N*/10 iodine added, one would expect more to be oxidised by the full dose of hypoiodite (5 cc. alkali) than by 3.0 cc. If only one component is oxidised, the small increments may be comparatively saturated with this component and each effect oxidation with rapidity comparable with that effected by the full dose.

Lastly, it should be noted that the high oxidation effected in this way without excess of alkali ever being present disproves the hypothesis that the oxidation of fructose in the iodimetric titration is due to a Lobry de Bruyn transformation.

Oxidation could be carried much beyond the 18 % recorded in Table III. To 0.081 g. fructose in 50 cc. were added 20 cc. of *N*/10 iodine and 0.5 cc. of

$N/2$ NaOH at varying time intervals as shown in Table VI. When 4 cc. of alkali had been added, *i.e.* just sufficient to absorb free iodine, to some flasks another addition of 20 cc. iodine was made and the process continued. The same procedure was repeated after 8 and 12 cc. of alkali had been added. Other flasks were treated with no more iodine after certain stages. In every case, after the last addition of iodine had been absorbed by alkali, two further increments of alkali were added at intervals to complete the reaction and then titration was performed in the usual way. The results are shown in Table VI. The percentage

Table VI.

0.5 cc. alkali/10 minutes		0.5 cc. alkali/30 minutes		0.5 cc. alkali/60 minutes	
Number of additions of 20 cc. $N/10$ iodine	% oxidation	Number of additions of 20 cc. $N/10$ iodine	% oxidation	Number of additions of 20 cc. $N/10$ iodine	% oxidation
1	10.4	1	19.5	1	23.6
2	20.6	2	40.6	2	61.1
3	40.1	3	77.3	3	98.9
		4	98.3		

fructose oxidised is calculated on the assumption that one atom of oxygen is absorbed by one fructose molecule. It is to be expected that, especially in the cases where the alkali additions were more frequent, a certain amount of unchanged hypiodite might accumulate. This would account for the rate of oxidation of fructose increasing as the additions of NaOH and iodine proceed.

These experiments were repeated, using 0.081 g. of fructose in 10 cc. instead of 50 cc.

Table VII.

0.5 cc. alkali/30 minutes		0.5 cc. alkali/60 minutes	
Number of additions of 20 cc. $N/10$ iodine	% oxidation	Number of additions of 20 cc. $N/10$ iodine	% oxidation
1	26.4	1	33.5
2	60.1	2	77.2
3	94.7	3	125.2
4	128.4		

It is evident that the oxidation proceeds beyond the insertion of one oxygen atom.

(4) *Effect of fructose concentration.*

If the hypothesis put forward in the preceding section is correct, then certain conclusions can be drawn. For example, in a dilute solution of fructose, varying within the limits of concentration 0.08–2 %, the amount of oxidisable component will be approximately proportional to the total fructose concentration. Under an appropriate set of oxidising conditions, the percentage fructose oxidised should be independent of the fructose concentration. To test this deduction, 20 cc. $N/10$ iodine were pipetted into 50 cc. of fructose solution containing 0.08–0.5 g. of sugar. 5 cc. of 0.5 N NaOH were added at the rate of 0.5 cc./30 mins. The mixture was acidified 1 hour after the last alkali addition, thus allowing a total oxidation period of 5.5 hours. The results obtained at 20° are given in Table VIII.

A second experiment designed for another purpose (*vide* Section (6)) gave similar results. Here the materials used were 0.081–0.5 g. fructose in 10 cc.

Table VIII.

Final fructose concentration (%)	Weight of fructose (g.)	Oxidation	
		cc. <i>N</i> /20 iodine	%
0.108	0.081	4.44	24.7
0.333	0.250	10.06	18.1
0.500	0.375	12.90	15.5
0.666	0.500	15.76	14.2

water, 6.66 cc. of 0.3 *N* iodine in BaI₂, 6.25 cc. of 0.4 *N* baryta added at a rate of 0.5 cc. per 30 mins. (total volume 22.91 cc.). Acidification was effected after oxidation had ceased with 10 cc. *N* HCl.

Table IX.

Final fructose concentration (%)	Weight of fructose (g.)	Oxidation	
		cc. <i>N</i> /20 iodine	%
0.354	0.081	6.18	34.3
1.092	0.250	14.01	25.3
1.747	0.400	19.83	22.3
2.184	0.500	23.64	21.3

The quantity of hypiodite maintained in these experiments (Tables VIII and IX) was of the order of the equivalent of 0.5 cc. *N*/2, which is equivalent to 0.0225 g. of glucose. If the whole of the fructose could react with hypiodite, there would be an overwhelming excess of fructose in all cases, and one would not expect much difference in the actual amounts oxidised in any one series. The whole or most of the hypiodite should be used up in oxidation and comparatively little would form iodate. However, the results in Tables VIII and IX support the hypothesis that only one component, a small one, of the fructose solution is oxidised. Some of the hypiodite is lost as iodate, the amount used for oxidation is largely a function of concentration of the oxidisable component. It is seen that, although the maximum fructose concentration is approximately 6 times as large as the minimum, the percentage oxidation varies by only 10–13 %. Indeed, if the minimum fructose concentrations of 0.108 in Table VIII, and of 0.354 in Table IX be disregarded, since here the excess of oxidising agent over percentage fructose oxidised is very large, the variation amounts to only 4 %.

(5) Control experiments.

In addition to such control experiments as have been mentioned it was shown that:

(a) there is only a negligible loss of oxygen from the hypiodite solutions as used in these experiments when maintained at temperatures from 1 to 35° for 24 hours;

(b) addition of the alkali at the rate of 0.5 cc. of *N*/2 per half hour made no appreciable difference in the oxidation of glucose, an increase from 100 to 103 % being the maximum observed;

(c) iodate exerted a negligible oxidising effect on fructose, and a slightly larger but small one on glucose;

(d) free iodine, as used in these experiments, but without addition of any alkali, did not oxidise fructose to any appreciable extent;

(e) excess of alkali such as was used in any experiment other than that of Table II had no effect on the optical rotation of fructose at 17° in 20 hours: when NaOH was present at *N*/14 concentration as in Exp. 2 (Table II) the observed rotation fell from –8.1° to –7.9° in a few minutes, to –7.45° in 1½ hours and to –6.55° after 20 hours (the oxidation results of Table II them-

selves pointed to the same conclusion, *viz.* that some change such as enolisation was taking place);

(f) oxidation of 0.072 g. of glucose under the conditions of Table I (Series A and B) showed complete oxidation (1 g. glucose = 1.41 g. iodine) almost attained in 8 hours at 1°, fully attained in 20 mins. at 15° and 35°.

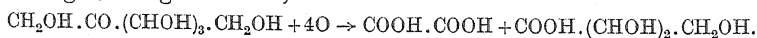
(6) *Identification of the products of oxidation of fructose.*

For this purpose, oxidation of fructose was effected by 0.3 *N* iodine in BaI₂ and 0.4 *N* baryta added in small increments. (Compare Goebel's procedure [1927, 2] for the preparation of hexonic acids.) Preliminary experiments (results in Table IX) had shown that the conditions adopted would be most favourable. 666 cc. of 0.3 *N* iodine were added to 40 g. of fructose dissolved in 1000 cc. of water. 625 cc. of 0.4 *N* baryta were added at the rate of 50 cc./30 mins. The temperature of working was 21–22°. 1 hour after the last alkali addition, the solution was filtered free of barium iodate and the exact amount of Ba in 10 cc. of the solution accurately determined gravimetrically. The whole of the barium in the residual solution was precipitated by the theoretical amount of 5 *N* sulphuric acid. 150 g. of pure lead carbonate were stirred in to decompose free hydriodic acid. After filtering, the clear solution was evaporated at 55°/15 mm. to 350 cc., the whole of the residual free iodine passing over in the first few minutes. The liquid during concentration remained slightly on the acid side of neutrality. After cooling, the precipitate of lead iodide which usually appeared during evaporation was filtered off and the clear filtrate made neutral to phenolphthalein by addition of clear baryta. 2 vols. of 95 % alcohol were now added and the flocculum so obtained was allowed to stand overnight and was then centrifuged off, washed with alcohol and dried *in vacuo* and at 100° for 3 hours. The white product thus obtained was not very soluble in water, reduced warm acid KMnO₄ but gave no reduction with Fehling's solution. The Ba content was determined and the bulk of the salt decomposed with the exact amount of *N*/10 sulphuric acid. After evaporation to a syrup *in vacuo*, a little acetone was added and after standing, crystals of oxalic acid appeared, melting at 97–98°. These were dissolved in alcohol, phenylhydrazine in alcohol was added, and on scratching the sides of the tube crystals of oxalic acid phenylhydrazone appeared in a few moments. These were filtered off, washed with hot 95 % alcohol, and after drying, melted at 176.5°. When mixed with an authentic specimen of the hydrazone (M.P. 179°), the mixed M.P. was 178°. The highest yield of crude barium oxalate obtained in several repeated experiments was 2.4 g., whilst the theoretical yield, calculated from Table IX, is 2.5 g.

A possible criticism of the above method is that, at the temperature used in the removal by evaporation of the free iodine, oxidation of unoxidised fructose by the latter may give rise to the oxalate eventually isolated. To eliminate any error on this account, another method was employed. Instead of precipitating the barium of the oxidation mixture with sulphuric acid, acidification was effected with *N* HCl, and free iodine removed by addition of concentrated thiosulphate. The solution was brought back to neutrality with 0.4 *N* baryta, and alcohol added until a flocculum appeared. This was again identified as barium oxalate in the manner previously described.

Oxidation of fructose by hypoiodite produces, therefore, oxalic acid, and, presumably, *d*-erythronic acid. An attempt at separation of the latter acid from unoxidised fructose was not successful.

Omitting the ring structure,

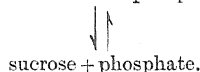
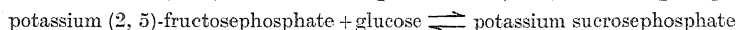


This oxidation is extremely remarkable, in that the small amount of oxidation which is induced by hypoiodite proceeds as far as the insertion of 4 oxygen atoms and the oxidation of a terminal alcoholic group. This fact strongly supports the hypothesis that only one component—an easily oxidisable component—undergoes oxidation.

DISCUSSION.

Whilst it is probable that one component only of fructose solution is oxidised by hypoiodite, the nature of this component is an open question. It may be the ketoform or it may be the labile (2, 5) form. In the previous paper (Bailey and Hopkins) the latter form, as split off from the sucrose molecule on hydrolysis by fructosaccharase, was shown to change to equilibrium fructose unimolecularly, the velocity constant being 0.3 (t in mins., base e , temperature 17°, p_H 4.6–6.1). The change is not so rapid as had been supposed. This observation supports the view that equilibrium fructose contains a small proportion of the (2, 5) form. The striking change in optical rotation with temperature can be best explained by a disturbance of the equilibrium between the (2, 6) and (2, 5) forms. Further evidence for the existence of the (2, 5) and open chain forms in equilibrium fructose solutions is summarised below:

(1) Oparin and Kurssanov [1931] have shown that fructosaccharase exerts a synthesising action on glucose and fructose in aqueous solution if phosphatase and potassium dihydrogen phosphate are present. They represent the reaction as (2, 6)-fructose \rightleftharpoons (2, 5)-fructose \rightleftharpoons potassium (2, 5)-fructosephosphate;



It is difficult to see how such a synthesis is to be achieved without the pre-existence in solution of (2, 5)-fructose, which on removal is replaced by the dynamic transformation of one or more remaining isomerides.

(2) According to Ohle [1927], the condensation of β -fructose with acetone in presence of mineral acid leads mainly to the production of α -di-*isopropylidene* fructose, *i.e.* without change of configuration or structure, whilst the liberated water converts a portion of the fructose into the butylene oxide form which is transformed into the corresponding *isopropylidene* ether. If fructose in neutral aqueous solution is changed by water in the same manner as during acetonisation, it follows, when the magnitude of specific rotation is taken into account, that the main chemical change during mutarotation is the transformation of the amylene oxide β -form into the butylene oxide β -form. This cannot occur without temporarily opening the oxygen bridge, with the production of the keto-form or its hydrate. The carbonyl absorption band is shown by concentrated fructose solutions.

(3) Pure methyl alcohol, freed from acetone, when mixed with less than 5 % fructose, exhibits a change in the sign of rotation 20 mins. after the addition of HCl. The syrup isolated is feebly dextrorotatory. Extraction with ethyl acetate leads to the separation of γ -methylfructoside mixed with small quantities of α - and β -(2, 6)-methylfructosides [Menzies, 1922].

Onslow, Kidd and West [1931], in a biochemical study of the senescence of apples, advanced tentatively the hypothesis that active fructose (presumably (2, 5)-fructose) is preferentially respired. Such a process is quite feasible, and could, moreover, proceed without the concomitant inversion of sucrose, *i.e.* it could proceed without a supply of freshly liberated or active fructose, if equilibrated fructose solutions contain this form of fructose. Whether the component

oxidised by hypiodite is the (2, 5) form or an active keto-form, is as yet impossible to decide. According to Isbell and Hudson [1932], ring forms can be directly oxidised. On the other hand, Lippich [1932] finds that the HCN-binding power of fructose is greater than that of glucose and is not wholly inhibited by strong alkali. Since HCN-binding power is a measure of CO groups, the active keto-form of fructose may thus be the form oxidised by hypiodite even at alkaline reactions. There is, however, a striking parallel between the *in vitro* experiments of Section (3), and the process which Onslow *et al.* presume to occur in the respiration of senescent apples. The fundamental nature of both processes may well be the oxidation (or respiration) of one component, accompanied by dynamic mutation of other components to produce more of the oxidisable isomeride.

SUMMARY.

1. The rate of oxidation of fructose by hypiodite under the conditions of Willstätter and Schudel's method for the estimation of aldoses increases with temperature to the point at which oxidation ceases. The extent of oxidation at this point, between the temperatures 1 and 35°, is minimum at 15°.
2. In presence of excess alkali, enolisation of the fructose occurs between 17 and 37° and renders it capable of oxidation by iodate. The oxidation thus becomes a progressive reaction which has no limit. At 1° enolisation is not induced, and oxidation rapidly reaches a limit lower than that obtained by the use of only a slight alkali excess.
3. A four to fivefold increase in the oxidation of fructose by hypiodite is obtained by adding the necessary alkali in small increments at definite time intervals. The extent of oxidation is an inverse function of the rate of alkali addition. Under specified conditions, the oxidation of fructose (using the iodine value 1.41 as for glucose) may exceed 100 %, on the basis of one atom of oxygen per molecule oxidised.
4. The percentage oxidation under arbitrary conditions of oxidation does not vary greatly with the fructose concentration.
5. The products of oxidation are oxalic and, presumably, *d*-erythronic acids.
6. A consideration of the factors influencing the kinetics of the oxidation of fructose by hypiodite leads to the hypothesis that only one component undergoes oxidation.

One of us (K. B.) is indebted to the Department of Scientific and Industrial Research for a Grant.

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CCLXVIII. THE CALCIUM OF WHOLE BLOOD, SERUM AND PLASMA IN HUMAN DISEASES, INCLUDING TETANY.

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(Received October 20th, 1933.)

THE work which forms the subject of this paper was suggested by the findings of Cameron and Moorhouse [1925], who stated that in experimental parathyroid tetany the calcium of the whole blood shows proportionately less fall than that of serum. They attributed this to the passage of a large amount of calcium into the fibrin clot in cases of tetany and based this view on the finding of a much higher calcium figure for citrated plasma than for serum in tetany. Sokolovitch [1931] has reported slightly higher figures for whole blood-calcium in human tetany than would be expected if the corpuscles contained no calcium and assumed that the results indicated a slight shift of calcium into the corpuscles in tetany. The effect observed by him was much smaller than that observed by Cameron and Moorhouse. Morgulis and Perley [1930] working on experimental parathyroid tetany, considered both possibilities—a shift of calcium into the corpuscles, and an abnormal passage of calcium into the coagulum—but could find no evidence for either phenomenon. The question of the passage of calcium into the corpuscles in tetany raises again the old controversy as to whether there is normally any calcium in red corpuscles. This point has been under discussion for nearly forty years, but there is still some divergence of opinion even among modern workers using accurate methods.

The present work was planned to determine whether any calcium exists in red corpuscles in human subjects with varying diseases including tetany, and whether any abnormal amount of calcium is to be found in the coagulum in cases of tetany.

Material.

The control observations were made for the most part in cases of renal or prostatic disease and a few other conditions.

Three cases of tetany were examined, *viz.* (1) H. D., age 22, tetany of unknown origin but probably not parathyroid tetany; (2) E. T., age 15, late rickets and tetany, and (3) A. Hi., age 35, chronic diarrhoea and tetany. No case of parathyroid tetany has been investigated from this standpoint.

Plan of experiments.

For experiments on the distribution of calcium between serum and corpuscles, defibrinated blood was used. The corpuscle volume was estimated by the haematocrit and the calcium estimated in serum and whole blood.

For experiments on the passage of calcium into the coagulum, comparisons were made between the plasma from citrated blood and the serum from defibrinated blood. Since citrate causes a shrinkage of corpuscles and a corresponding dilution of the plasma, it was necessary to make haematocrit determinations on both the citrated and defibrinated blood in order to determine the correction

needed for the calcium of citrated plasma. The collection of blood was made as follows. About 20–25 cc. of blood were collected in a flask containing glass beads, and the flask was shaken for a few seconds to ensure even admixture of corpuscles with plasma. Then, before the fibrin had begun to separate, about 10 cc. of blood were poured off into a tube containing not more than 0.1 g. sodium citrate and mixed. The remainder of the sample in the flask was defibrinated by further shaking.

In order to check the validity of the volume correction used in determining calcium in plasma, a few experiments were made in which a sample was first defibrinated and a part of the defibrinated blood subsequently treated with sodium citrate. The usual contraction of corpuscles was observed in the citrated samples. When 0.1 g. sodium citrate was added to 10 cc. blood the corpuscle volume in the citrated blood was, on an average, 38 % as against 45 % in defibrinated blood. The correction to be used was determined for each experiment and the corrected plasma figures agreed well with the serum figures. This result shows that the addition of sodium citrate in this amount causes no precipitation of calcium and no shift of calcium from plasma to corpuscles.

Analytical methods. For serum and plasma the method of direct precipitation of the calcium oxalate from the serum or plasma was used, and the analyses were made by the method of Kramer and Tisdall as described by Harrison [1930], except that at least 18 hours' precipitation was allowed for plasma. The precipitation of calcium is much slower for plasma than for serum. In the earlier part of the work half an hour was usually allowed for the precipitation from serum, and 18 hours at least for plasma. In some later experiments the serum was also left for 18 hours, but the results were the same. G. W. Clark [1921] emphasised the necessity of allowing several hours for the precipitation of calcium from plasma. E. P. Clark and Collip [1925] considered half an hour sufficient for serum.

For whole blood, the modified Alport method of Cameron and Moorhouse [1925] was used. In this method the blood is ashed in a platinum dish, and the iron and phosphate are removed from the ash solution before the calcium is precipitated as oxalate.

In all estimations samples of 2 cc. serum, plasma or whole blood were analysed in duplicate.

Estimations of corpuscle volume were made in capillary haematocrits, which were centrifuged at about 3000 r.p.m. until the corpuscle layer reached constant volume. When constant volume was reached, it was always noted that the corpuscle mass became transparent.

RESULTS.

Serum and corpuscles. Observations were made on fourteen patients with various diseases and the three patients with tetany. Very good agreement was found in most cases between the observed figure for whole blood and the figure for the serum-calcium calculated as mg. per 100 cc. whole blood. Where slight discrepancies occurred, the tendency was for the observed whole blood figures to be slightly lower than the calculated figures. The actual discrepancies between observed and calculated figures for whole blood were as follows (a positive sign indicates that the observed figure was higher than the calculated figure, and a negative sign the reverse discrepancy):

In the 3 cases of tetany, $-0.2, 0, 0$ mg. per 100 cc.

In control cases: ± 0.2 mg. per 100 cc. in 9 experiments.

± 0.2 to 0.5 mg. per 100 cc. in 2 experiments.

-0.5 to -1.0 mg. per 100 cc. in 3 experiments.

Agreement is therefore good in 14 experiments including those in cases of tetany, while three experiments show either a negative error in determining whole blood-calcium (which might be due to loss in the transferences) or a positive error in the serum determinations (which might be due to the use of direct precipitation). The results therefore show that there is no measurable amount

of calcium to be found in corpuscles by the technique adopted, and that no shift of calcium into corpuscles occurs in the types of human tetany examined.

Serum and plasma. The average results in the control experiments were as follows:

Serum-calcium	9.52 mg. per 100 cc.
Plasma-calcium (observed)	9.04 mg. per 100 cc.
Plasma-calcium (corrected for volume change)	10.05 mg. per 100 cc.
Calcium passing into the clot	0.53 mg. per 100 cc.

It will be seen that the serum-calcium and plasma-calcium are approximately equal, but the result is due to a partial balance of opposite effects—the dilution of the plasma due to the addition of citrate tends to lower the plasma figure, and the passage of a trace of calcium into the clot tends to lower the serum figure as compared with plasma.

In the three cases of tetany, no measurable passage of calcium into the clot was observed.

DISCUSSION.

These results in three cases of human tetany, not of parathyroid origin, are similar to the results of Morgulis and Perley [1930] in parathyroid tetany. No calcium passes into the corpuscles and no abnormal amount into the coagulum.

The question of the absence of calcium from normal corpuscles requires further comment. In reading the literature the main impression is of the number and variety of sources of error in the methods for calcium estimation. Almost every detail in every method has been the subject of debate, but these technical points will not be discussed in detail, because it seems impossible to harmonise all the results even when all the sources of error are considered. The technical problems are very fully dealt with by Guillaumin [1930].

There may be differences between different species, and there may be differences between adults and children, but to settle this point more work is needed, applying the same methods to blood from different sources. Almost all the work on human adults shows absence of any appreciable amount of calcium from corpuscles, but sometimes figures of about 3 mg. Ca per 100 cc. corpuscles, or even more, occur among the results of authors whose technical work appears very accurate. Some of these figures have been obtained by direct determinations on unwashed corpuscles and are therefore open to the objection that they include some calcium from the serum remaining in the corpuscle mass, but values from 3 to 6 mg. per 100 cc. corpuscles cannot be accounted for in this way.

SUMMARY.

Calcium was found to be absent from the red blood corpuscles in human subjects with various diseases including tetany.

No abnormal amount of calcium passed into the fibrin coagulum during clotting of the blood of human subjects with tetany.

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CCLXIX. THE TOTAL AND DIFFUSIBLE CALCIUM OF SERUM AND THE CALCIUM OF CEREBROSPINAL FLUID IN HUMAN CASES OF HYPOCALCAEMIA AND HYPERCALCAEMIA.

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(Received October 28th, 1933.)

CASES of hypocalcaemia may be divided into three groups. Firstly, there are the various types of low-calcium tetany. Secondly, there are cases of hypocalcaemia associated with low serum-protein, in which no symptoms of tetany occur; this condition may be present in nephrosis, chronic nephritis with oedema, kala-azar and some other conditions. Thirdly, there are cases of chronic nephritis with uraemia, in which the inorganic phosphorus of the serum is increased, and in which the serum-proteins may be normal or only moderately reduced; in some instances neuromuscular irritability and carpopedal spasm occur in addition to uraemic manifestations.

One would expect that in tetany a low $[Ca^{++}]$ would be the significant change. The ionic calcium may or may not account for the whole of the diffusible calcium of serum, but it must be included in the diffusible fraction. It would also be expected that when the serum-calcium falls in response to a rise in the inorganic phosphate, the diffusible fraction would be primarily affected, though the change in the diffusible fraction would lead to a secondary change in the protein-bound fraction as a result of the disturbance of equilibrium. On the other hand, where the serum-calcium falls as a result of a fall in the serum-protein, one would expect the protein-bound fraction to be reduced and the diffusible fraction normal, and the absence of tetany would thus be explained.

Now those authors who have used the concentration of calcium in the cerebrospinal fluid as a measure of the diffusible calcium of the serum have arrived at results which are inconsistent with the above views, for it has been shown that when the serum-calcium falls in tetany, the calcium of the cerebrospinal fluid is relatively little changed. Authors who have determined the diffusible calcium of the serum by ultrafiltration or dialysis have arrived at variable results, but, on the whole, work done on ultrafiltration of human sera has shown that the diffusible calcium is decreased in tetany and normal in cases of hypocalcaemia with low serum-protein. The view that the calcium of cerebrospinal fluid is equal to the diffusible calcium of serum has been attacked, but as yet only a few comparisons between the cerebrospinal fluid and the serum ultrafiltrate have been made in cases where the total serum-calcium is abnormal.

At the outset of the present work the intention was to make comparisons between the calcium of serum and of cerebrospinal fluid in conditions where the

serum-calcium was markedly raised or lowered, and this has been done in cases of tetany, uraemia, osteitis fibrosa and some other diseases. Later the scope of the work was extended, and the serum was analysed for total calcium, diffusible calcium, protein and phosphorus. It is unfortunate that some cases of tetany were examined before the experiments on ultrafiltration were begun.

METHODS.

Calcium was estimated by the method of Kramer and Tisdall as described by Harrison [1930], but the following technical points require comment. Firstly, the time allowed for precipitation of the calcium oxalate was in most instances more than 2 hours and often overnight. This was done as a precaution, though half an hour is probably adequate for the majority of sera. Secondly, the conditions of washing the precipitate of calcium oxalate must be considered. The precipitates obtained from cerebrospinal fluids or from ultrafiltrates do not pack closely enough to be left undisturbed by decantation, and removal of the supernatant with a pipette is therefore preferred. In analyses of pure calcium chloride solutions it was found that the soluble oxalate was completely removed by the second washing, and that a third washing caused no loss of calcium oxalate (ordinarily the residue was twice washed). In these and all other determinations the "blank" value was obtained by taking a little of the last supernatant fluid (comparable with that left with the final residue) and treating it in the same way as the residue. In this way correction is made for any soluble oxalate remaining with the calcium oxalate. The "blank" value was almost constantly 0.03 cc. *N*/100 permanganate.

Wherever possible, 4 cc. of cerebrospinal fluid or ultrafiltrate were used, but in some cases it was not possible to obtain so much ultrafiltrate, and some of the figures for diffusible calcium are based on single determinations on 2 cc. fluid. The errors likely to occur under these conditions are not great enough to affect the main significance of the results.

Phosphate. Inorganic phosphate was estimated in serum or oxalate-plasma by the method of Briggs [1922]. The time elapsing between collection of the blood and removal of the plasma from the corpuscles was not more than half an hour and was usually about 10 to 15 minutes.

Serum-proteins. Only total protein estimations have been made in the present series of cases. Some of these were made by the micro-Kjeldahl method using Pregl's apparatus [Harrison, 1930] but the majority by the colorimetric method in the form used by Greenberg [1929, 1].

In a preliminary comparison of these two methods, using sera and exudate fluids in which the ratio of albumin to globulin varied through a wide range, it was found that the results agreed well for total protein, but that considerable discrepancies sometimes occurred in the determination of the ratio of albumin to globulin. Tuchman and Sobotka [1932] have obtained similar results in comparing the Kjeldahl method with the colorimetric method as described by Wu.

Albumin and globulin have different tyrosine equivalents in the colorimetric method, but in spite of this there was a very constant relation between the total protein-nitrogen as determined by the Kjeldahl method and the tyrosine equivalent of total protein in the colorimetric method. Greenberg's factor, 1 mg. tyrosine \equiv 16.0 mg. total protein, was found to hold good in spite of variations in the ratio of albumin to globulin, and it was therefore considered justifiable to make total protein estimations by the colorimetric method without separating the albumin and globulin fractions.

In the two cases where Bence-Jones protein was present in the serum, the total protein was estimated by the Kjeldahl method.

Urea was determined by the method of Archer and Robb [1925].

Ultrafiltration. Collodion sacs were cast inside tubes 10 cm. in length and 1.6 cm. in diameter. The solution used was 3 % pyroxylin in alcohol and ether (40 parts alcohol to 60 parts ether) and 3 cc. ethylene glycol were added to 100 cc. of the pyroxylin solution. The sacs retained protein completely, as was shown by testing the ultrafiltrates with salicylsulphonic acid. The permeability of the sacs to calcium was checked by filtering the same sample of serum through the different sacs and showing that all the ultrafiltrates contained the same concentration of calcium. The permeability of the sacs was very constant and regular, and sacs which had been in use some time gave the same results as new ones.

Table I.

Case	Age	Sex	Diagnosis	Date	Cerebro-spinal fluid-calcium mg. per 100 cc.	Serum-calcium mg. per 100 cc.			Diff. fusible Ca % of total	Serum-protein %	Plasma-phosphate (inorganic) mg. P per 100 cc.	Blood-urea mg. per 100 cc.
						Diff. fusible	Total	Non-dif. fusible				
C. F.	18	F	Osteitis fibrosa with parathyroid hyperplasia	14. ix. 32	—	—	19.2	—	—	1.70	26	
				19. ix. 32	6.4	—	17.8	—	—	—		
				11. x. 32	—	9.2	17.8	8.6	—	—		
				17. x. 32	—	9.4	15.6	6.2	—	—		
S. H.	52	F	Osteitis fibrosa with parathyroid hyperplasia	28. x. 32	5.9	9.3	16.1	6.8	—	—	—	
				2. vi. 33	—	6.9	13.0	6.1	1.68	—		
				19. vii. 33	6.3	8.4	14.7	6.3	—	—		
				25. vii. 33	6.9	7.2	16.9	9.7	43	12.6	4.2	120
A. Ho. M. L.	54 to 13	F	Multiple myelomatosis Late rickets	6. v. 31	—	—	15.6	—	—	2.8	38	
				10. vii. 31	5.7	—	16.0	—	—	—	—	—
				8. v. 33	—	6.3	10.8	4.5	58	2.35	—	—
				—	—	5.9	10.8	4.9	55	7.8	4.28	66
J. R. R. F.	28 22	M F	Acute nephritis Carpopedal spasm and dwarfism. Hysteria	31. v. 32	5.6	—	10.5	—	—	4.72	—	
				5. v. 33	—	5.3	10.8	5.5	49	6.4	3.58	30
M. S. H. H.	— 55	F M	? Tetany. Radium menopause Multiple myelomatosis	21. iii. 33	—	6.0	10.7	4.7	56	6.1	2.72	48
				13. ii. 33	—	5.9	10.6	4.7	56	6.5	4.67	102
K. L. D. O.	42 28	F F	Congenital cystic kidney Convulsions during pregnancy	21. iv. 33	—	5.8	10.4	4.6	56	6.2	2.86	30
				21. iii. 33	—	6.0	10.3	4.3	58	6.4	3.22	—
W. McG. C. T.	16 13	M F	? Osteitis fibrosa Osteitis fibrosa	8. vi. 33	—	5.1	9.8	4.7	52	6.2	3.07	—
				2. v. 33	—	5.4	9.6	4.2	56	5.9	3.26	25
R. B. M. R.	22 —	F M	Hysteria and carpal spasm Progressive muscular atrophy	27. ix. 33	—	4.6	9.5	4.9	48	6.2	1.91	—
				29. vii. 33	4.7	—	9.2	—	—	—	—	—
R. H.	11	M	Subacute nephritis	16. i. 33	5.7	5.7	9.1	3.4	63	—	—	90
A. T.	30	F	Chronic nephritis	8. ii. 33	—	4.7	9.1	4.4	52	5.8	4.60	203
T. H.	33	M	Congenital cystic kidney	3. iv. 33	—	5.2	8.5	3.3	61	5.5	7.05	282
G. G.	58	M	Chronic nephritis	11. xi. 32	6.2	6.0	8.4	2.4	71	6.1	12.70	400

P. M.	56	M	Chronic nephritis	14. iii. 33	—	5.9	8.1	2.2	73	4.5	3.04	30
J. McP.	56	F	Myxoedema, pyelonephritis	22. x. 32	—	4.9	8.1	3.2	61	5.1	3.92	70
J. T.	33	M	Nephritis	26. v. 33	—	5.3	7.7	2.4	69	5.3	6.60	475
J. St.	18 to 19	M	Acute to subacute nephritis	22. x. 32 23. ix. 33 25. ix. 33 4. x. 33	— 5.2 — 4.9	4.5 — 2.8 3.2	8.0 — 7.3 6.0	3.5 — 4.5 2.8	56 — 38 53	4.9 — 4.6 —	7.3 — 12.6 12.2	112 — 198 —
M. H.	17	F	Acute to subacute nephritis	30. i. 33 7. ii. 33 20. ii. 33 27. ii. 33 28. iv. 33	— — — — —	5.7 4.4 4.0 4.0 3.8	7.8 7.3 6.7 6.8 7.1	2.1 2.9 2.7 2.8 3.3	73 60 60 59 54	5.7 5.3 5.2 5.4 5.3	8.15 14.2 13.8 13.1 5.98	215 — 368 320 337
F. W.	35	F	Chronic nephritis	22. ii. 32 4. iv. 32	4.5 —	— —	6.7 7.0	— —	— —	— 5.6	— 2.32	21
H. D.	22	F	Tetany. (In attack)	20. v. 32	4.9	—	6.4	—	—	—	3.22	—
E. T.	15	F	Late rickets and tetany	19. iv. 33	5.7	4.0	6.4	2.4	63	5.4	28.1	690
T. Y.	43	M	Chronic nephritis, Tetany	25. iv. 33	4.3	3.0	5.4	2.4	56	5.0	3.20	—
A. Hi.	34	F	Chronic diarrhoea and tetany	9. v. 33 16. v. 33 2. vi. 33	— 4.9 4.6	3.9 4.5 4.1	6.3 6.9 5.4	2.4 2.4 1.3	62 65 76	— — —	— — —	— — —
J. Sh.	36	M	Chronic nephritis	21. x. 32	5.3	2.8	6.2	3.4	45	7.0	26.2	410
G. P.	7	M	Chronic nephritis, uraemia and tetany	1. v. 31 6. v. 31 14. v. 31	— 5.3 4.8	— — —	5.6 — 6.1	— — —	— — —	— — —	10.4 — 6.6	156 — —
R. A.	39	M	Chronic nephritis	2. ii. 32	4.7	—	5.1	—	—	—	—	480
E. F.	39	F	Exophthalmic goitre. Postoperative tetany	19. vii. 32	4.4	—	4.4	—	—	—	6.3	—

NOTES

C. F. and S. H. In each case hyperplastic parathyroid glands were found at operation, and the blood chemistry returned to normal after their removal.
A. Ho. Bence Jones protein was present in serum and urine. *Post mortem* examination showed multiple bone tumours (plasma-cell myeloma), gross renal disease, and normal parathyroid glands.

M. L. An unusual case of late rickets of unknown origin and resistant to treatment.

R. F. Carpal spasm, believed to be due to hysteria and probably not alkalosis tetany.

R. B. Carpal spasm certainly due to hysteria. The electrical excitability of the motor nerves was normal.

H. D. Idiopathic tetany, probably not of parathyroid origin.

E. F. Tetany after thyroidectomy for exophthalmic goitre. The observations were made two months after the operation.

In the course of ultrafiltration, the sacs tended to dry above the level of the serum contained in them. In time this made it necessary to discard old sacs. The drying caused them to become less permeable, in the sense that filtration became unduly slow, though it did not appear to alter the composition of the ultrafiltrate. The sacs were stored in saline solution in the refrigerator. Before use they were dried as completely as possible with filter-paper and rinsed with a little of the serum to be filtered. The first drops which formed when ultrafiltration began were blotted off the sac with filter-paper. Ultrafiltration was carried out under a diminished pressure of about 150mm. mercury in the apparatus described by Greenberg and Gunther [1929]. The rate of filtration was such that with new sacs containing 5 to 6 cc. of serum about 1 cc. ultrafiltrate was collected per hour.

The figures obtained at the moderate pressure used in this method are comparable with those of the majority of other authors who have worked on human material. High pressure ultrafiltration may give higher figures for diffusible calcium [Nicholas, 1932].

Ultrafiltration is preferred to compensation dialysis as a method of estimating the diffusible calcium, because dialysis against any external fluid which does not contain the same concentration of free calcium as the serum is likely to cause some disturbance of the equilibrium between protein-bound calcium and diffusible calcium, with a consequent change in the distribution ratio. The concentration of the fluid inside the sac during ultrafiltration does not lead to any such disturbance of the distribution ratio, provided that the filtration is not too long continued [Stewart and Percival, 1928, 1]. In the course of the present work it has been noted that the serum inside the sac can be concentrated to at least half the original volume without causing any change in the composition of the ultrafiltrate.

RESULTS.

The results are given in Table I.

Normally the total serum-calcium ranges between 9 and 11 mg. per 100 cc.; the cerebrospinal fluid-calcium varies from 4.5 to 5.5 mg. per 100 cc. and the diffusible serum-calcium is approximately equal to the cerebrospinal fluid-calcium.

The present series of observations includes nineteen strictly simultaneous estimations of total serum-calcium and cerebrospinal fluid-calcium, in seventeen of which the serum-calcium is abnormal. The serum-calcium values range from 4.4 mg. per 100 cc. to 17.8 mg. per 100 cc., but the whole range of variation in the cerebrospinal fluid-calcium is from 4.4 to 6.9 mg. per 100 cc. The calcium of cerebrospinal fluid never definitely falls below normal in hypocalcaemia and only rises slightly above normal in hypercalcaemia.

There are three comparisons between the diffusible serum-calcium and cerebrospinal fluid-calcium in cases of hypercalcaemia. In the two cases of hyperparathyroidism, the diffusible calcium is markedly raised and forms a normal proportion of the total calcium, and there is a striking difference between the calcium of the ultrafiltrate and that of the cerebrospinal fluid. The case of C. F. shows that the discrepancy cannot be due to a delay in attaining equilibrium between serum and cerebrospinal fluid. When the observation was made on Oct. 28th, it was known that the total serum-calcium had ranged between 15.6 and 19.2 mg. per 100 cc. for more than six weeks, and in fact it had been rising for months. The diffusible calcium had been observed to remain in the region of 9.3 mg. per 100 cc. for seventeen days before the final observation, yet the cerebrospinal fluid-calcium was 5.9 mg. per 100 cc., only slightly above normal.

In the case of A. Ho., the hypercalcaemia was mainly due to a rise in the non-diffusible fraction of the serum-calcium, and there was also a rise in the serum-protein as a result of the presence of Bence-Jones protein in the serum. There was a moderate rise in the diffusible serum-calcium, and very little difference between the ultrafiltrable calcium and cerebrospinal fluid-calcium.

When the total serum-calcium is markedly reduced in tetany or in uraemia, the diffusible calcium of the serum is also low and falls definitely below the cerebrospinal fluid-calcium (6 observations).

In uraemia, a moderate fall of serum-calcium (9.0 to 7.5 mg. per 100 cc.) is usually associated with normal figures for diffusible calcium. This may occur in spite of moderate rises in the inorganic phosphate of the plasma (6.6 to 12.7 mg. P per 100 cc.). Higher phosphate figures are associated with more marked hypocalcaemia and a fall in the diffusible calcium of the serum. In most cases the serum-proteins are low, but the distribution of calcium between the diffusible and non-diffusible fractions does not bear a regular relationship to protein and phosphate figures.

There are only two cases of hypocalcaemia with low serum-protein and normal plasma-phosphate (P. M. and J. McP.); in both of these the diffusible calcium is normal.

Observations on diffusible calcium have been made in one case of tetany with low serum-calcium. The diffusible calcium was 4.1 mg. per 100 cc. or lower at all times when symptoms were present; on one occasion when symptoms were absent the diffusible calcium was 4.5 mg. per 100 cc.

The series includes two cases of carpopedal spasm of unknown causation, with normal figures for both total and diffusible calcium in the serum (R. F. and R. B.). These are not regarded as cases of tetany. Greenberg and Gunther [1931] mention three similar cases of "hysteria and carpopedal spasm."

DISCUSSION.

The relationship between the total calcium of serum and cerebrospinal fluid.

Parallel determinations of calcium in serum and cerebrospinal fluid in experimental animals have been made by Cameron and Moorhouse [1925], Merritt and Bauer [1931, 2], Morgulis and Perley [1930] and Hertz [1930]. By the work of these authors it has been established that in parathyroidectomised animals the calcium of the cerebrospinal fluid is only slightly lowered in spite of the hypocalcaemia, and that when the total serum-calcium falls to the level of 4 to 5 mg. per 100 cc. the figures for calcium in serum and cerebrospinal fluid become approximately equal. On the other hand, when hypercalcaemia is induced by administration of parathormone, the cerebrospinal fluid-calcium is only slightly raised, and figures of 12 to 17.2 mg. per 100 cc. in serum have been recorded in association with figures of 5.2 to 6.8 mg. per 100 cc. in cerebrospinal fluid. These results are shown in Fig. 1. Similar, though rather more variable results were found when hypercalcaemia was induced by continuous intravenous injections of calcium salts [Morgulis and Perley, 1930]. The findings in the present series of human cases agree well with those recorded in experimental hypo- and hyper-calcaemia.

Parallel observations of calcium in serum and cerebrospinal fluid in various human diseases have been made by Cantarow [1929, 1, 2], Leicher [1922], Pincus and Kramer [1923], Berenczy [1929], Merritt and Bauer [1931, 1, 2], Critchley and O'Flynn [1924], Lennox and Allen [1930], Kral *et al.* [1929], Weston and Howard [1922], Parhon and Ornstein [1930], Hamilton [1925], Halverson and Bergeim [1917], McCance and Watchorn [1931; 1932, 1], and Nourse *et al.* [1925]. The vast majority of the observations of these authors were made in cases where the total serum-calcium was normal or only slightly raised or lowered. The data obtained in meningitis must be considered separately, for, as Merritt and Bauer [1931, 1] have pointed out, the cerebrospinal fluid-calcium

may rise above normal in such cases. The rise in protein in the cerebrospinal fluid in inflammatory conditions will cause some alteration in the distribution of calcium between plasma and cerebrospinal fluid, but there may be other factors involved. Observations in such cases are not comparable with those in which the meninges are normal and ought not to be used as a basis for the study of the normal equilibrium between plasma and cerebrospinal fluid. The data of McCance and Watchorn [1932, 1] for cases of meningitis include some definitely high figures for cerebrospinal fluid-calcium (6.5 to 7.9 mg. per 100 cc.), but the majority of the results fall between 5.2 and 6.5 mg. per 100 cc. McCance and Watchorn [1931] also record a number of high figures for cerebrospinal fluid-calcium in cases of general paralysis, cerebral haemorrhage and cerebral arterial sclerosis.

A very large number of analyses have been made in other human diseases—including non-inflammatory diseases of the nervous system, mental diseases and a wide variety of diseases not affecting the nervous system. In all these conditions the cerebrospinal fluid-calcium ranges between 4.5 and 6.5 mg. per 100 cc. with only occasional exceptions.

Parhon and Ornstein [1930] reported low figures for the calcium of cerebrospinal fluid in epilepsy, but this is not confirmed by other authors.

Data for hypocalcaemia in human subjects are given by Cantarow [1929, 2] in three cases of bronchial asthma; by Merritt and Bauer [1931, 2] in three cases of tetany; by Leicher [1922] in one case of tetany; by Lennox and Allen [1930] in three cases of tetany; and by Nourse *et al.* [1925] in seventeen cases of tetany. The results show that it is extremely unusual for the calcium of the cerebrospinal fluid to fall below 4 mg. per 100 cc.

In Fig. 2 are assembled the data for tetany and for various other human diseases, collected from the literature, and including the observations of the present paper. The data for normal subjects and for cases of mental and nervous disease are excluded as being too numerous to plot. Also Cantarow's results [1929, 1] cannot be shown owing to his method of recording them.

Observations on the calcium of cerebrospinal fluid after parathormone administration in human subjects have been made by Merritt and Bauer [1931, 2] in cases of epilepsy, by Cantarow [1929, 2] in cases of epilepsy and bronchial asthma, and by Berenczy [1929] in various diseases. In the cases of Merritt and Bauer the calcium of cerebrospinal fluid remained normal in spite of hypercalcaemia, and in many of Cantarow's cases the same result was obtained, though some showed definite rises in the cerebrospinal fluid-calcium. All Berenczy's cases showed rises in the cerebrospinal fluid-calcium, but in all it was abnormally high before parathormone was given.

The relation between the calcium of cerebrospinal fluid and the diffusible serum-calcium.

Normally the figures for calcium are approximately the same in cerebrospinal fluid and in ultrafiltrate. Very few comparisons have been made in cases where the serum-calcium is abnormal. McCance and Watchorn [1931], and Greenberg [1929, 2] have made comparisons, and found some discrepancies greater than can be accounted for by experimental error. In some cases the ultrafiltrate contained more calcium than the cerebrospinal fluid, in others the reverse discrepancy occurred, but very few of the figures were definitely abnormal. Greenberg considers that "when the blood-calcium is at a normal stable level, there is an approximate approach to an equilibrium between blood and spinal fluid, and the spinal fluid-calcium is then a close measure of the diffusible

calcium; but on the other hand, when the blood constituents are undergoing marked fluctuations the spinal fluid changes do not keep pace." On this basis the relatively constant figures for cerebrospinal fluid-calcium in animals subjected to parathyroidectomy or treated with parathyroid hormone are explained. The present work shows that there must be some other factor involved beside the time factor—in the case of C. F. the cerebrospinal fluid failed to come into equilibrium with the blood after prolonged hypercalcaemia. Hertz [1930] has shown that in parathyroid tetany in animals, the diffusible calcium may be low while the cerebrospinal fluid-calcium is normal. The results of the present paper show similar discrepancies.

The observation that in tetany the cerebrospinal fluid-calcium is not significantly lowered, whilst the diffusible serum-calcium is lowered, may explain the fact that the nervous symptoms of tetany are peripheral and not central. A reduction in the $[Ca^{++}]$ of the fluid in contact with the central nervous system has been shown to cause convulsions of central origin [Huggins and Hastings, 1933.]

The distribution of calcium between the diffusible and non-diffusible fractions in protein solutions and in serum.

For the purpose of the present discussion it will be assumed that the non-diffusible calcium is bound to protein. A number of observations show that the diffusible and non-diffusible fractions of the serum-calcium are not to be regarded as independent entities but are in equilibrium with each other. The removal of calcium ions by precipitation with oxalate leads to a dissociation of the protein-bound fraction, so that the whole of the serum-calcium can be precipitated as oxalate. Similarly, the addition of citrate to the serum causes the whole of the serum-calcium to become diffusible [Stewart and Percival, 1928, 2], presumably owing to the removal of calcium ions by the formation of a non-ionised complex. The serum-calcium can be completely removed by dialysis if very large volumes of external saline solution are used; this shows that removal of the diffusible calcium disturbs the equilibrium and causes dissociation of the protein-bound fraction [Loeb and Nichols, 1923]. The balance between the two fractions may also be disturbed by the addition of more calcium in diffusible form; the added calcium becomes distributed between both fractions so that at the new equilibrium the non-diffusible fraction forms approximately the same proportion of the total as before. This occurs both *in vivo* and *in vitro* [Smith and Sternberger, 1932].

The conditions of this equilibrium have been studied in dialysis systems by Marrack and Thacker [1926] and by Loeb and Nichols [1923; 1925; 1927, 1, 2]. This work has shown that the proportion of calcium which becomes bound to protein, in serum or other protein-containing mixtures, depends upon the following factors: the protein concentration; the nature of the protein; the calcium concentration in the external solution; the hydrogen ion concentration; the chloride concentration; and the temperature. Some of these factors will vary in different pathological conditions, and there may be other significant factors as yet unrecognised.

If the protein concentration varies, other factors being constant, the ratio of the protein-bound calcium to the diffusible calcium varies directly as the protein concentration.

If the protein, p_H and chloride concentrations are constant, and the calcium varies, there is a regular relationship between the protein-bound calcium and the calcium of the external solution, as is shown by the experiments of Loeb and

Nichols [1927, 1]. The regularity of these results is shown in Fig. 3, where the results of Loeb and Nichols are plotted in a manner comparable with the other charts in the present paper—*viz.* the total calcium values in the protein solution are plotted as abscissae, and the calcium values of the aqueous solutions as

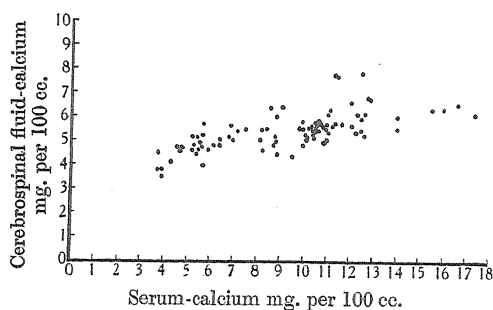


Fig. 1.

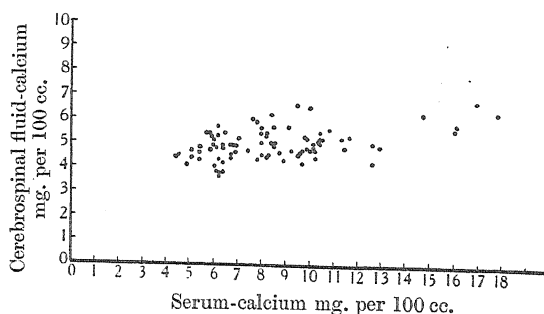


Fig. 2.

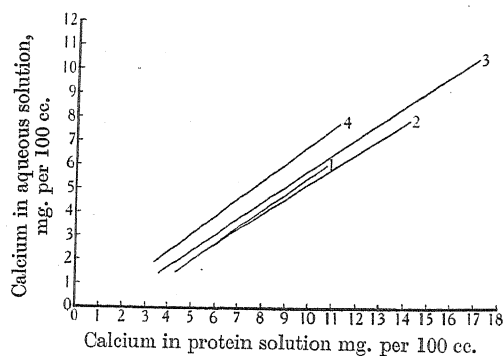


Fig. 3.

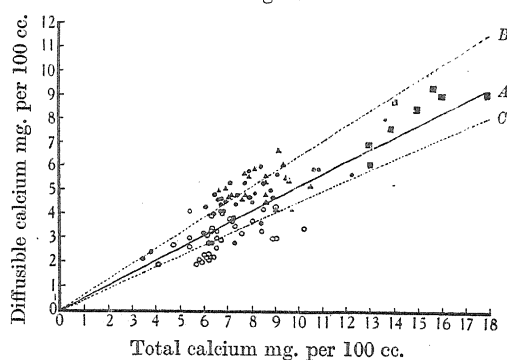


Fig. 4.

Fig. 1. Relation between serum-calcium and cerebrospinal fluid-calcium in animals at different levels of parathyroid function (parathyroidectomy; normal; and parathyroid-treated). Data collected from the literature.

Fig. 2. Relation between serum-calcium and cerebrospinal fluid-calcium in human tetany and other human diseases. Data collected from the literature together with those of the present paper.

Fig. 3. The distribution of calcium between serum and external fluid in a dialysis system. Data of Loeb and Nichols. Each straight line represents the results obtained on one serum sample when dialysed against external fluids of varying calcium content.

Fig. 4. Relation between total and diffusible serum-calcium in various human diseases. Data collected from the literature including the present paper.

- Cases of tetany.
- Cases of uraemia.
- ▲ Serum-proteins below 6 % in cases other than uraemia or tetany.
- Hyperparathyroidism.

ordinates. For each serum the result is a straight line, but the straight lines do not pass through the origin—with low calcium concentrations a greater proportion of calcium is protein-bound. Greenberg and Gunther [1929] have pointed out that these results are in accordance with Langmuir's adsorption isotherm.

A change in the hydrogen ion concentration towards the acid side will cause a decrease in the amount of protein-bound calcium, whereas a change to the alkaline side will cause an increase. If the reaction is at the isoelectric point of the proteins the whole of the calcium becomes diffusible [Loeb and Nichols, 1925; Pincus *et al.*, 1926]. The changes likely to occur in pathological conditions will probably cause only slight alterations in the proportions of diffusible and non-diffusible calcium. Marrack and Thacker [1926] state that a change from p_H 7.4 to 7.8 will cause the diffusible calcium to fall from 5.4 to 4.5 mg. per 100 cc. They make the suggestion that alkalosis tetany may be due to this effect, but Greenberg and Gunther [1931] have found that in alkalosis tetany the diffusible calcium is normal. Hertz [1929] found a slight, but scarcely significant, fall in the diffusible calcium in this condition.

A fall in chloride concentration causes an increase in the proportion of calcium bound to protein, and the effect is large enough to be of importance in pathological conditions. The figures of Loeb and Nichols [1927, 1] indicate that if the total calcium remained steady at 13.0 mg. per 100 cc. one would expect that a fall in chloride concentration from approximately 130 to approximately 30 *mM* would cause a fall of diffusible calcium from 8 mg. per 100 cc. to approximately 5 mg. per 100 cc.

In this discussion the diffusible fraction has been treated as a single entity. If it in fact includes two components, an ionised portion and a non-ionised diffusible complex, then there will be two balanced reactions to consider—between ionic calcium and protein-bound calcium, and between the ionised and non-ionised fractions of the diffusible calcium. Such subdivisions are still a matter of controversy. Benjamin and Hess [1933] subdivide both diffusible and non-diffusible fractions according to the amount removed by adsorbing agents.

Total and diffusible calcium in pathological conditions.

The distribution of calcium in human sera in various diseases has been studied by McCance and Watchorn [1931; 1932, 2], Benjamin and Hess [1933], Greenberg and Gunther [1930, 1, 2, 3; 1931; 1932], Greenberg [1929, 1, 2], Liu [1927; 1928], Kirk and King [1926], Pincus *et al.* [1926], Snell [1930], Weill [1932, 1, 2] and Hertz [1929].

For the purposes of the present discussion the results of these authors, together with those of the present paper, have been grouped as follows: (1) tetany, excluding alkalosis tetany, 28 cases; (2) uraemia, 33 cases; (3) serum-proteins below 6 % in cases other than uraemia and tetany, 20 cases; (4) pregnancy, 16 cases; (5) hyperparathyroidism, 5 cases, and (6) all conditions not included in the preceding categories, 180 cases. Some observations have been omitted owing to difficulty in classifying them. The last group serves as a control group. The "normal" diffusible calcium as judged from these data may be taken as 4.5 to 6.5 mg. per 100 cc. In 90 % of the control cases the diffusible calcium forms 45 to 65 % of the total and is on the average 52 %.

Data in cases of pregnancy vary. McCance and Watchorn [1932, 2] found in most instances an unusually high proportion of diffusible calcium; Kirk and King [1926] recorded the reverse effect.

The data from the other groups of human cases collected from the literature, together with those reported in the present paper, are assembled in Fig. 4. Here the total serum-calcium values are plotted as abscissae and the diffusible calcium values as ordinates. The line *OA* is drawn through the origin and the point corresponding to total calcium 10.0, diffusible calcium 5.2; any observations where the diffusible calcium forms 52 % of the total will fall on this line.

which marks the average percentage of diffusible calcium in the control cases. Similarly *OC* and *OB* represent 45 % and 65 % respectively, and mark the range within which 90 % of the "control" data fall. The actual "control" data are not plotted, but the lines *OA*, *OB*, and *OC* are derived from them and serve as guides to show whether, in the other cases, the diffusible calcium keeps the normal proportion or not. Points falling above *OB* indicate a disproportionately low non-diffusible fraction, and points falling below *OC* indicate a disproportionately low diffusible fraction.

In the group of cases where the serum-protein is below 6 % (plotted in Fig. 4 as solid triangles), the diffusible calcium is almost always normal, and when the total calcium is low, the protein-bound fraction is reduced. This is most clearly shown in those cases where the serum-protein is very low [Liu, 1927].

McCance and Watchorn [1932, 2] state that the protein concentration of the serum has no effect on the proportion of non-diffusible calcium. Their data, however, were obtained on sera in the majority of which the proteins were normal. It appears that so long as the variations in the serum-protein fall within the normal range, the relationship between protein and protein-bound calcium is obscured by other factors affecting the equilibrium, but when the protein is markedly reduced, the protein concentration becomes the predominant factor.

The cases of uraemia (plotted as solid circles in Fig. 4) are collected from the data of McCance and Watchorn [1932, 2], Pincus *et al.* [1926], Hertz [1929] and the present paper. The proportions of diffusible and non-diffusible calcium vary widely, and although in general low serum-protein is accompanied by a high proportion of diffusible calcium, and marked phosphate retention causes a fall in both total and diffusible calcium, it is not possible to explain the figures entirely in terms of variations in protein and phosphate. The effects of acidosis and of chloride retention probably play a part also. The phosphate retention alone should not cause any disturbance of the normal distribution ratio [Marrack and Thacker, 1926].

It is of interest that a normal diffusible calcium concentration may exist in spite of phosphate retention—examples of this occur in the data of Pincus *et al.* and in the present paper. In the more severe degrees of hypocalcaemia the absolute value of the diffusible calcium falls, and in such cases tetany may occur. No definite "tetany level" of diffusible calcium seems to exist in uraemic cases.

In the 15 cases of tetany (plotted in Fig. 4 as open circles) the diffusible calcium ranges from 2.0 to 4.2. There are four observations where the points fall above *OA*; three of these are the data from the case A. Hi. of the present paper, where the serum-protein was below normal. The majority of the other observations show a disproportionate reduction in the diffusible calcium as compared with the total calcium. This would be expected in hypocalcaemia with normal serum-protein (see Fig. 3). Some of the cases of tetany described by Greenberg and Gunther [1931] show a definite reduction in the diffusible calcium associated with a normal total calcium figure. The variation in the proportion of diffusible to total calcium may possibly be connected with variations in chloride metabolism. According to Morris *et al.* [1931] there are abnormalities of chloride metabolism in tetany. Although the proportion of diffusible calcium to total calcium is so variable in tetany, these observations on human cases show very definitely that the absolute value of the diffusible calcium is always subnormal; all cases of tetany show diffusible calcium values of 4.2 mg. per 100 cc. or lower. It is striking that a horizontal line drawn across Fig. 4 at the level of 4.2 mg. diffusible calcium per 100 cc. precisely separates the tetany cases from the cases

in the "low protein" group. (The data on human tetany are derived from: Greenberg and Gunther [1931]; Liu [1927; 1928]; Pincus *et al.* [1926]; Snell [1930], Hertz [1929] and the present paper.)

The data for diffusible calcium in experimental parathyroid tetany are much less regular than those just discussed. Such observations have been made by Von Meysenbug and McCann [1921], Cruikshank [1923], Pincus *et al.* [1926], Reed [1928], Hertz [1930], Benjamin and Hess [1933], Snell [1930] and Moritz [1925].

The results of Von Meysenbug and McCann, and Cruikshank were obtained using compensation dialysis as a method of determining the diffusible calcium and Lyman's nephelometric method for the calcium analyses. (Lyman's method has been criticised as giving irregular results.) The majority of the data of the remaining authors, who used ultrafiltration, show that the diffusible calcium falls in parathyroid tetany though the proportion of the diffusible to the total calcium is variable.

The data obtained for dogs at different levels of parathyroid function (normals, parathyroidectomised dogs and dogs treated with parathyroid extract) are collected in Fig. 5, which is compiled from the data of Pincus *et al.*, Reed, Hertz, Benjamin and Hess, and Snell. The results of Moritz for rabbits are similar.

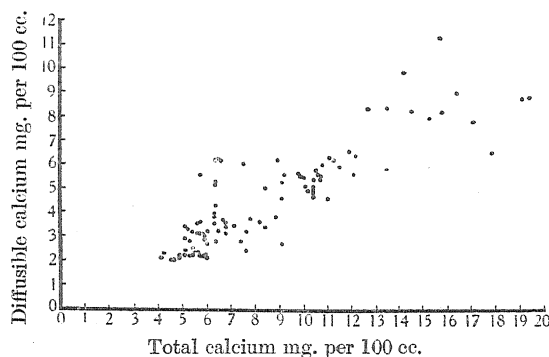


Fig. 5. Relation between total and diffusible calcium in dogs at different levels of parathyroid function. (Parathyroidectomy; normal; parathyroid-treated).
Data collected from the literature.

There are observations in five cases of human hyperparathyroidism—one recorded by Benjamin and Hess [1933], two by Snell [1930] and two in the present paper. These show that the total and diffusible calcium both rise, maintaining their normal proportions (Fig. 4). The data in animal experiments are very variable (Fig. 5). Probably in the human subjects, suffering from chronic disease, the metabolic conditions are more constant and regular than in animals subjected to experimental procedures. The observations in hypo- and hyper-parathyroidism do not suggest that the action of the parathyroid hormone in any way affects the distribution of calcium between the diffusible and non-diffusible fractions. It seems probable that whatever may be the concentration of the diffusible calcium, and whatever the factors that determine it, the concentration of the protein-bound calcium will adjust itself to come into equilibrium with the diffusible calcium, the particular distribution ratio being determined by the nature and quantity of the protein, the hydrogen ion concentration, and the chloride concentration. No description of the variations in the distribution ratio can be complete without a knowledge of all these factors.

SUMMARY.

1. In human cases of hypocalcaemia and hypercalcaemia, the calcium of the cerebrospinal fluid remains relatively constant in spite of very wide variations in serum-calcium.

2. The cerebrospinal fluid-calcium cannot be taken as a measure of the diffusible calcium of serum. In hyperparathyroidism the diffusible calcium of the serum is greater than the cerebrospinal fluid-calcium; in tetany and in some cases of uraemia the diffusible calcium falls below the cerebrospinal fluid-calcium.

3. In hypocalcaemia associated with low serum-protein and normal inorganic phosphate, the diffusible calcium is normal.

4. In the hypocalcaemia of uraemia, the diffusible calcium of serum may be normal if the reduction in total calcium is only moderate. With the more severe degrees of hypocalcaemia, the diffusible calcium falls below normal, and in such cases tetany may occur.

5. In tetany, the diffusible calcium falls below normal; the relation of diffusible to total calcium is variable.

6. In hyperparathyroidism, the diffusible calcium rises, and the normal ratio of diffusible to total calcium is maintained.

I wish to express my thanks to members of the Medical and Surgical Staff of the Royal Victoria Infirmary and the Newcastle General Hospital for kindly giving me the opportunity to carry out the investigations here recorded.

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CCLXX. STUDIES ON THE EXPERIMENTAL PRODUCTION OF SIMPLE GOITRE¹.

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(Received November 7th, 1933.)

It is generally agreed at the present time that the immediate cause of simple hyperplastic goitre is an iodine deficiency, whereby the thyroid gland becomes hyperplastic in an attempt to manufacture an adequate amount of its iodine-containing hormone. This is amply proved by the work of Marine who has shown that the administration of adequate amounts of iodine will prevent the occurrence of thyroid hyperplasia no matter what the goitrogenic agent. Iodine deficiency may be absolute or relative, or a combination of the two. In absolute iodine deficiency there is a deficient intake; in relative deficiency the intake is normal, but either (1) the absorption of iodine from the alimentary tract is impaired, or (2) the tissues for some reason utilise larger amounts of the thyroid hormone than the gland can normally supply without enlarging. It is this second factor which is of importance in studying the fundamental cause of simple goitre, for it is probably the essential cause of those thyroid enlargements which occur at puberty, during pregnancy and lactation and at the menopause.

The production of goitre by cabbage feeding.

Chesney, Clawson and Webster [1928] discovered that their stock rabbits, maintained on a diet consisting of a daily ration of fresh cabbage, a ration of oats three times a week and one of hay once a week developed large hyperplastic goitres. This was subsequently confirmed by Marine *et al.* [1929], and by McCarrison [1931]. It was further shown by Marine *et al.* [1929] and Webster *et al.* [1931] that the production of the thyroid hyperplasia was not due to an absolute iodine deficiency but to a powerful goitrogenic agent contained in the cabbage. Samples of cabbage grown in different parts of the country had approximately the same iodine content but varied greatly in their goitrogenic potency, and goitrogenic cabbage when dried in a current of air at room temperature lost its power of producing goitre [Marine *et al.*, 1930]. The observation that the goitrogenic power of cabbage is not dependent on an absolute iodine deficiency has recently been confirmed by Hercus and Aitken [1933] in New Zealand. An active sample of cabbage may produce in rabbits a thyroid enlargement two or three times its normal size in three weeks. Steaming the cabbage increases its activity [Marine *et al.*, 1929]. The goitrogenic substance in cabbage is extractable with ether [Baumann *et al.*, 1931]. Various vegetables of the *Brassica* group, such as Brussels sprouts (*B. oleracea*, var. *bullata gemmifera*) and cauliflower (*B. oleracea botrytis*) were found to produce thyroid hyperplasia approximately to the same degree as cabbage (*B. oleracea capitata*) [Marine *et al.*, 1929].

¹ Communicated to the Medical Research Society on October 20th, 1933.

Realising the importance of being able to produce goitre in rabbits by means of cabbage feeding, we made the attempt in London in 1929, reproducing as nearly as possible the conditions to which Chesney's animals were subjected. The animals used were young Dutch rabbits, their weights at the beginning of the experiment ranging from 1 to 2 kg. They were housed in separate cages and were not exposed to direct sunlight. Wood shavings were used as bedding and were renewed once a week. The cages were cleaned out once a week. Six rabbits received as their diet a daily ration of fresh cabbage obtained from a greengrocer, a ration of whole oats three times a week and hay once a week. They received no water to drink. Six rabbits as controls received a daily ration of hay, bran and greenstuff such as pea-pods. Before the animals were put under experimental conditions, the neck was opened under ether anaesthesia and the thyroid found to be normal in size and appearance. A small portion of the gland removed for section was normal. On February 12th the animals were put under experimental conditions which were maintained until December 3rd, a period of 295 days. Their nutrition and growth were good, but at the end of this time their thyroid glands were normal in size and in histological appearance.

Marine *et al.* [1929], Webster *et al.* [1931], McCarrison [1931] and McCarrison and Madhava [1932-33] demonstrated later the occurrence of seasonal variations in the goitrogenic activity of cabbage, cabbage maturing in the autumn and winter being active and cabbage maturing in the spring and summer being practically inactive. This factor could not account for the negative results in this experiment, as the animals received both varieties, and winter cabbage was fed for the two months preceding the end of the experiment. It was considered at this time that English cabbage was non-goitrogenic.

Having seen in Marine's laboratory the considerable thyroid enlargement which may be produced in 3 weeks by cabbage feeding, and taking into consideration the fact that the goitrogenic power of cabbage shows considerable variations in different years [Webster *et al.*, 1931], we repeated these experiments in the spring and summer of 1933. The cabbage used was summer cabbage and was obtained from Cornwall. Negative results were obtained in a batch of four rabbits aged 9 weeks which were fed with steamed whole cabbage in amounts equivalent to 75 calories per kg. per day (the food value of cabbage being calculated as 3.1 g. equivalent to 1 calorie), with the addition of 17 g. of whole oats and 20 g. of hay twice weekly.

Although we did not expect positive results with summer cabbage, from the negative results which we had been obtaining with other known goitrogenic agents we began to suspect that our previous failure to produce thyroid hyperplasia by means of cabbage feeding might have been due to an antigoitrogenic property of the hay and oats which supplemented the animals' diet. Consequently we fed a batch of eight rabbits, aged 2 to 4 months, on fresh cabbage (from Buckinghamshire) daily; four received in addition 35 g. of whole oats daily, and four 35 g. of rolled oats daily. They received no hay and no water. At the end of 21 days those on cabbage and rolled oats had marked thyroid hyperplasia and enlargement; those on cabbage and whole oats had hyperplasia and enlargement, but less marked (Table I). Three animals with marked thyroid hyperplasia then continued with their diet of cabbage and oats, with the addition however of hay, for a further 21 days. At the end of this time their thyroids had involuted. This was not due to the inactivity of this sample of cabbage, since in a concurrent experiment it produced thyroid hyperplasia.

These experiments show fairly definitely that the hay and whole oats obtained in this country have an antigoitrogenic effect, and may explain our

Table I. *The effect of hay, whole oats and rolled oats on the production of goitre in rabbits by feeding with equivalent amounts of cabbage daily for 21 days.*

Hay and whole oats					Whole oats. No hay					Rolled oats. No hay				
No.	Sex	Age weeks	Weight in g.	Condition of thyroid	No.	Sex	Age months	Weight in g.	Condition of thyroid	No.	Sex	Age months	Weight in g.	Condition of thyroid
71	F	9	1095	—	75	F	3½	1155	— +	69	M	4	1420	+
72	M	9	1017	—	77	F	3	1252	+	80	M	3	1217	+
75	F	9	844	—	91	M	2	807	— +	107	M	2½	1300	+
76	F	9	972	—	92	M	2	947	— +	106	M	2	892	— +

+ = about twice normal size, marked hyperplasia. — + = slightly enlarged, moderate hyperplasia. — = normal.

previous failure to produce thyroid hyperplasia by cabbage feeding. It is of interest that the batch of cabbage used in this experiment matured in the early summer and yet in the absence of hay was markedly goitrogenic. A subsequent batch of cabbage, however, obtained from the same district in the late summer during and just after a period of considerable drought showed little if any goitrogenic power. This was of value in demonstrating that the rolled oats had no goitrogenic effect. It is interesting to note in this connection that McCarrison and Madhava [1932-33] found that the goitrogenic potency of cabbage attained its maximum after the heavy autumn rains.

The production of goitre by methyl cyanide.

It was suggested by Marine *et al.* [1929] that the goitrogenic agent in cabbage acts by depressing some oxidation system, an effect which the thyroid attempts to overcome by producing a larger amount of its iodine-containing hormone with consequent hyperplasia. In studying the chemistry of the Cruciferae, to which the cabbage family belongs, it was found that the presence of a mustard oil (*isothiocyanate*) was a specific characteristic of this group of plants [Gilde-meister and Hoffmann, 1916]. Weith [1873] had observed that when a mustard oil is distilled under suitable conditions it is converted into its corresponding cyanide (nitrile), and about the same time Hoffmann [1874] had succeeded in isolating organic cyanides from several of the Cruciferae. These observations, and the fact that cyanide is a powerful depressor of tissue oxidation led Marine *et al.* [1932] to determine the effect of the daily subcutaneous injection of small doses of various organic cyanides on the thyroid gland of young rabbits maintained on a diet of alfalfa hay and whole oats. Striking thyroid hyperplasia and enlargement were obtained after 21 days in about 70 % of the animals by the daily subcutaneous injection of 0.1 to 0.15 cc. doses of methyl cyanide, the least toxic of the nitriles. This has been confirmed by McCarrison [1933]. Several other organic cyanides produced thyroid reactions to a less degree. The production of thyroid hyperplasia by means of organic cyanides is dependent entirely on the gradual liberation of hydrocyanic acid in the organism, and it was found that organic cyanides which are relatively stable, such as phenyl cyanide (C_6H_5CN), produced no thyroid reaction. Further, animals such as chickens, in which hydrocyanic acid is liberated only to a very small degree even from such a nitrile as methyl cyanide, failed to develop thyroid hyperplasia [Spence, 1933]. The production of thyroid hyperplasia by means of cyanides could be prevented by the administration of iodine [Marine *et al.*, 1932], and is an illustration of the importance of a relative iodine deficiency in the aetiology of goitre. The particular nitrile which may be present in cabbage has not been isolated; in McCarrison's

laboratory acid hydrolysis of cabbage yielded 6.3 mg. of cyanogen per kg., which did not necessarily represent the total amount present [Ranganathan, 1933].

In endeavouring to repeat the production of thyroid hyperplasia by methyl cyanide in London we were again impressed with the importance of diet in the prevention of goitre, a fact which McCarrison has repeatedly stressed. Four rabbits, aged $2\frac{1}{2}$ to 3 months and weighing from 600 to 2550 g. received daily subcutaneous injections of methyl cyanide in doses ranging from 0.06 to 0.16 cc. They were maintained on a diet of hay, whole oats and tap water. At the end of 24 days their thyroid glands were normal. Because of these negative results it was considered that the diet which they were receiving might be markedly antigoitrogenic. To test this hypothesis a batch of 24 rabbits, aged 2 to $3\frac{1}{2}$ months, were given 0.1 cc. of methyl cyanide daily, but were divided into four groups, which received various modifications in their diet. All were kept on about 20 g. of hay daily, but group I received daily in addition 35 g. of whole oats and tap water, group II, 35 g. of rolled oats and distilled water, group III, 35 g. of rolled oats and tap water, and group IV, 35 g. of whole oats and distilled water (Table II). Those on whole oats and tap water again had normal thyroid glands at the end of 21 days; of those on rolled oats and distilled water, three had marked thyroid

Table II. *The effect of methyl cyanide on the thyroid gland of rabbits fed on various diets. (Period, 21 days.)*

Hay, whole oats, tap water						Hay, rolled oats, distilled water					
No.	Sex	Age months	Weight in g.	Daily dose of methyl cyanide (subcut.) cc.	Condition of thyroid	No.	Sex	Age months	Weight in g.	Daily dose of methyl cyanide (subcut.) cc.	Condition of thyroid
55	F	$3\frac{1}{2}$	1325	0.1	—	59	M	$3\frac{1}{2}$	1004	0.1	(—)*
65	F	3	1165	"	—	64	F	3	1167	"	+
72	M	$2\frac{1}{2}$	1132	"	—	70	F	$2\frac{1}{2}$	1075	"	++
71	F	$2\frac{1}{2}$	1370	"	—?	73	M	$2\frac{1}{2}$	1069	"	++
78	M	2	992	"	—+	77	F	2	1102	"	—
79	F	2	1330	"	—	80	M	2	1082	"	—
Controls receiving no cyanide						74	F	$2\frac{1}{2}$	1115	Nil	—
						75	F	$2\frac{1}{2}$	953	"	—
						81	M	2	1175	"	—?
						82	M	2	1140	"	—?
Hay, rolled oats, tap water						Hay, whole oats, distilled water					
No.	Sex	Age months	Weight in g.	Daily dose of methyl cyanide (subcut.) cc.	Condition of thyroid	No.	Sex	Age months	Weight in g.	Daily dose of methyl cyanide (subcut.) cc.	Condition of thyroid
72	M	$3\frac{1}{2}$	1132	0.1	—+	71	F	$3\frac{1}{2}$	1537	0.1	—
68	M	3	1217	"	—	66	F	3	1175	"	—?
69	M	3	1175	"	—	67	F	3	1079	"	—+
100	M	$2\frac{1}{2}$	1157	"	—	97	F	$2\frac{1}{2}$	1157	"	—?
101	F	$2\frac{1}{2}$	1130	"	—?	99	M	$2\frac{1}{2}$	1187	"	—+
86	M	2	992	0.075	—	98	M	2	852	0.075	—
Controls receiving no cyanide						76	F	$2\frac{1}{2}$	1125	Nil	—?
						60	F	$3\frac{1}{2}$	937	Nil	—

++ = more than three times normal size, marked hyperplasia. —+ = slightly enlarged, moderate hyperplasia.
 +± = more than twice normal size, marked hyperplasia. —? = possibly slightly enlarged, possibly slight hyperplasia.
 + = about twice normal size, marked hyperplasia. — = normal.

* Died after four days.

enlargement, two had normal glands and the sixth died during the experiment. The thyroid responses in the other two groups on rolled oats and tap water, and on whole oats and distilled water, were either slight or negative. Four control animals of the same age and breed, which received no cyanide, but a diet of hay, rolled oats and distilled water, had normal thyroid glands. The three rabbits in group II which had marked thyroid hyperplasia were then given whole oats and tap water, instead of rolled oats and distilled water, and continued with their cyanide injections for a further 27 days. At the end of this time their thyroid glands had involuted.

There is thus no doubt that whole oats and tap water are definitely antagonistic to the goitrogenic action of methyl cyanide. From our cabbage experiments there is also evidence that better thyroid reactions could be obtained with cyanide if the hay were eliminated. Experiments are proceeding on these lines, for in order ultimately to test the antigoitrogenic power of various substances it is essential to have a reliable method of producing thyroid hyperplasia, and with this in view it is consequently of importance to use a diet which is not anti-goitrogenic. On the other hand, it is preferable to use only one goitrogenic agent, and therefore in producing thyroid hyperplasia by means of methyl cyanide or any other goitrogenic substance, care should be taken to determine that the diet is not goitrogenic. The diet used should be one which McCarrison terms neutral, and this will vary in different localities. Cabbage feeding does not form a reliable method of producing thyroid hyperplasia, because as mentioned previously the goitrogenic activity of cabbage varies so considerably.

With regard to the nature of the antigoitrogenic substance, or substances, in hay, whole oats and tap water, we have not made determinations of the iodine content, as it does not lie within the scope of our present research. The iodine content of London tap water is approximately 4γ (0.004 mg.) per litre [Houston, 1929], which is insufficient by itself to prevent thyroid hyperplasia under the above experimental conditions. In considering the possibility of any difference in the iodine contents of whole oats and of rolled oats, it is unlikely that iodine is lost during the process of producing rolled oats. This process consists essentially in removing the husk by crushing and then passing the oats between hot rollers. Marine *et al.* [1933] have recently drawn attention to the occurrence in plants of antigoitrous substances other than iodine. Such substances have to be taken into account in considering the antigoitrogenic action of various foodstuffs.

The relation between the production of thyroid hyperplasia by methyl cyanide (acetonitrile) and the Hunt acetonitrile reaction.

Objections to the hypothesis concerning the mode of production of thyroid hyperplasia by means of methyl cyanide have been raised [Thomson and Collip, 1933] on the grounds that it does not appear to run parallel with the work of Hunt [1923], who showed that the administration of thyroid substance and thyroxine diminished the resistance to acetonitrile of all animals tested, except the mouse. Hunt explained the diminished resistance to acetonitrile following the ingestion of thyroid substance by the increased metabolism, whereby hydrocyanic acid was liberated more rapidly from the molecule of acetonitrile with consequently greater toxic effects. There is no evidence to refute this hypothesis, and we are in complete agreement with it. Superficially it appears that if, according to Marine's theory with which we also concur, thyroid hormone is antagonistic to the action of cyanide, then one would expect the administration of thyroid hormone to increase the resistance to acetonitrile. This, however, does not necessarily follow, for it would hold only if the

antagonism between thyroid hormone and cyanide were a direct one. The antagonism between the two substances is more complicated.

The experiments of Hunt on the diminished resistance to acetonitrile in hyperthyroid animals do not contradict the hypothesis of Marine and his collaborators on the mode of production of thyroid hyperplasia by means of acetonitrile, because the two series of experiments are not comparable, in that they involve two entirely different mechanisms. If thyroid hormone be given before the acetonitrile, so that the organism has an increased metabolism, then one would expect the resistance to acetonitrile to be diminished, as Hunt has explained. If, on the other hand, acetonitrile be given without the previous administration of thyroid, then there is a depression of tissue metabolism which the thyroid endeavours to overcome.

SUMMARY.

1. Thyroid hyperplasia has been produced in rabbits by feeding cabbage which was grown in England, although this cabbage matured in the early summer. Cabbage which matured in the later summer during a period of drought was practically inactive.
2. Previous failures to produce thyroid hyperplasia by means of cabbage were due not necessarily to the inactivity of the cabbage, but to the presence of antigoitrogenic substances in the hay and oats with which the animals' diet was supplemented.
3. The production of thyroid hyperplasia in rabbits by means of the daily administration of small doses of methyl cyanide (acetonitrile) has been confirmed.
4. In attempting to produce thyroid hyperplasia by means of goitrogenic agents, it is essential that the animals' diet be not antigoitrogenic. It is important that their diet should be what McCarrison terms neutral.
5. The relation between the production of thyroid hyperplasia by acetonitrile and the Hunt acetonitrile reaction is discussed.

We wish to thank Prof. F. R. Fraser for the interest he has taken in this work. One of us (A. W. S.) is greatly indebted to the Medical Research Council for personal and expenses grants.

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CCLXXI. THE TRANSMISSION OF VITAMIN A FROM PARENTS TO YOUNG IN MAMMALS.

II. THE CAROTENE AND VITAMIN A CONTENT OF COW'S COLOSTRUM.

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(Received October 12th, 1933.)

IN an earlier paper [Dann, 1932, 2] an account was given of experimental work devised to ascertain the channels through which young mammals receive supplies of vitamin A up to the time of weaning. The placenta, the colostrum and the milk are the three available channels; but of their relative importance and the factors controlling the efficiency of each we know almost nothing. The experiments previously described were made upon rats and rabbits, and unfortunately in these species it was not possible to distinguish between the colostrum and the milk. It is therefore necessary to examine the colostrum and milk of other species in order to find their relative importance in supplying vitamin A to the suckling. At the same time it is to be expected that variation will occur between different species, and so it will be necessary to examine the colostrum of a number of species before drawing any general conclusions about the importance of colostrum as a vitamin A carrier. In this paper a preliminary study of cow's colostrum is described.

EXPERIMENTAL.

The experimental animals. The work to be described consists of a series of measurements of the vitamin A and carotene content of the colostrum and milk of individual cows of a herd of dairy shorthorns belonging to Cambridge University Farm. The composition and management of the herd have been described by Mansfield and Garner [1931], and for the present purpose it is only necessary to recapitulate the following details of the feeding of the animals. The herd grazes collectively in summer and in the winter receives hay collectively in covered yards. During the winter roots are given to the animals individually and all the year round concentrated foods are given individually to each animal in proportion to its milk yield. The roots given are marrow kale, sugar beet pulp or mangolds; the concentrates vary according to the market price of the constituents, but a typical concentrate contains crushed beans, flaked maize, crushed oats, ground nut cake, cottonseed cake, bran and steam bone flour. Thus the carotene of the cow's diet (on which it is wholly dependent for carotene and vitamin A in the colostrum and milk) is chiefly supplied by the grass or hay, to a small extent by the roots, and much less still by the concentrates.

In the management of lactation it must be noted that the cows are allowed a dry period of two months before calving, and after birth a calf is allowed to suck

¹ Medical Research Scholar of the Worshipful Company of Grocers.

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for four days before it is weaned and the parent milked. Throughout lactation the cows are milked morning and evening and the best milkers in the afternoon

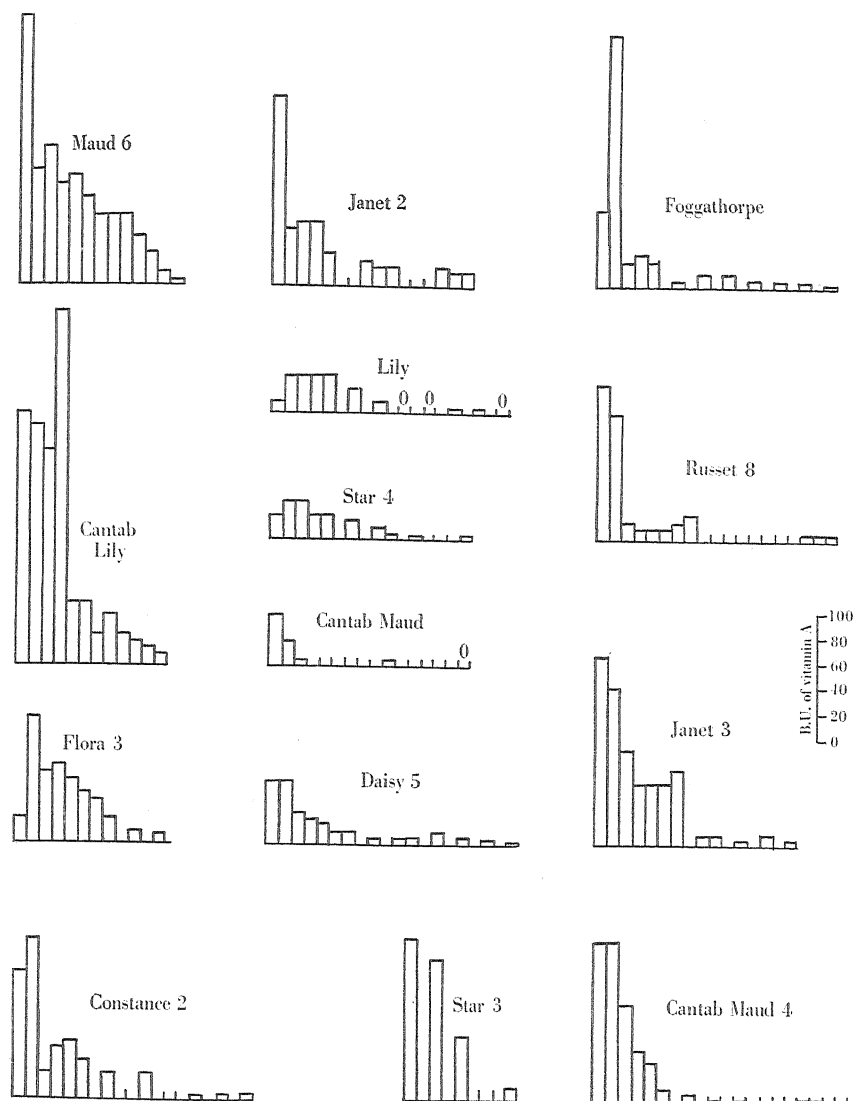


Fig. 1. The vitamin A concentration in colostrum and milk of individual cows. In each diagram the columns refer to successive samples of colostrum and milk obtained at 12-hourly intervals; the left-hand column represents the first sample taken just after calving. The height of the closed columns indicates the amount of vitamin A in B.U. (on the scale given) contained in 10 g. of the sample. Columns not closed at the top indicate that the 12-hourly sample was not assayed, except where the figure 0 is placed above the column to show that no measurable amount of vitamin A was contained in 10 g. of the sample.

also. All samples examined in this work were taken at morning or evening milking time except the first sample from each animal, which was taken within an hour after calving.

Examination of the samples of colostrum and milk: Each sample of colostrum and milk was examined by colorimetric methods for carotene and vitamin A. So far as the writer is aware, no method of separating the vitamin A from milk in a way suitable for subsequent application of the Carr-Price test has been

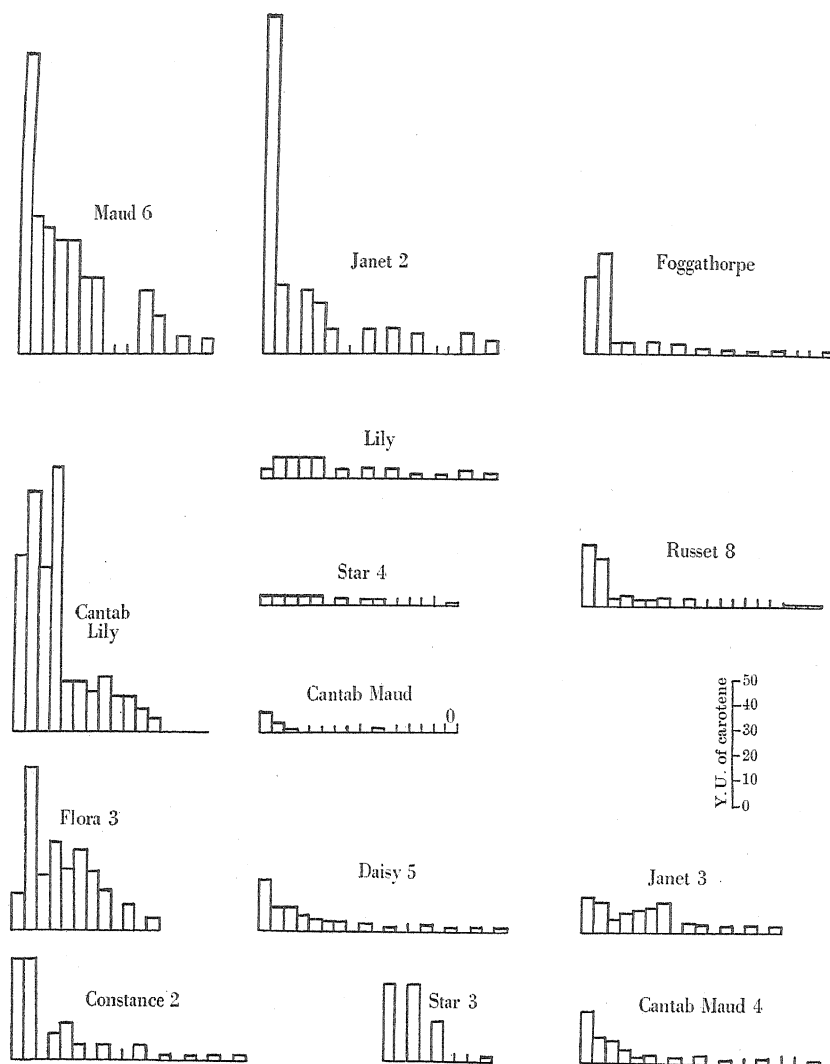


Fig. 2. The carotene concentration in colostrum and milk of individual cows.
Arrangement of diagrams exactly as in Fig. 1.

described. In extracting the carotene and vitamin A it was however found possible to apply the simple alkali digestion method used for animal tissues, with slight modification. The strength of potash used in digesting tissues (usually about 5 %) is insufficient to digest the proteins of milk in a reasonable time. As it has been shown [Dann, 1932, I] that even strong alcoholic

potash does not destroy vitamin A over long periods of time, the effect of strong aqueous potash in digesting the milk samples was tried. This has proved quite satisfactory, and tests have shown that no measurable destruction of vitamin A occurs over digestion periods up to twelve hours under the conditions described. The following procedure was used throughout. 10 g. of the sample were weighed into a 150 cc. conical flask and 10 cc. of 66 % KOH (*w/v*) added, after which the flask was placed in an oven at 100° and left for 2–4 hours, when digestion had usually proceeded sufficiently far. The flask was then cooled and its contents poured into a separating funnel together with 10 cc. of alcohol and 30 cc. of ether. On shaking the flask, the fat, carotene and vitamin A present in the digest passed into the ether layer, which was then separated, washed, dried and evaporated in the usual way. The resultant fatty residue was dissolved in chloroform and its carotene content determined by direct measurement of the yellow colour of the solution in a Lovibond tintometer. This procedure estimates xanthophyll and carotene together, but in the light of recent work [*cf.* Gillam *et al.*, 1933] it is clear that the error in carotene determination in butter or milk-fat caused by the presence of the xanthophyll is on the average about 7 %; it is therefore not worth while separating the xanthophyll as the error inherent in the colorimetric method used is greater than that due to the presence of the xanthophyll. The vitamin A content of the chloroform solution was determined by the method of Carr and Price, the depth of blue colour produced with SbCl_3 in the Lovibond tintometer being estimated. In order to make sure that the error due to the presence of xanthophyll was unimportant in carotene estimations on colostrum, the xanthophyll content of three samples of colostrum was determined by the method of Gillam *et al.* The first samples of colostrum obtained from Maud 6th, Cantab Lily and Russet 8th yielded a mixture of pigments in which the xanthophyll accounted for 7.6, 6.9 and 7.2 % respectively of the total colour. The ratio of xanthophyll to carotene therefore appears to be the same in colostrum as in milk.

In this way a large number of samples have been examined and the data are presented graphically in Figs. 1 and 2. In Fig. 1 the vitamin A content of each sample is given in blue units (B.U.) of vitamin A and in Fig. 2 the carotene contents are given in yellow units (Y.U.) of carotene. For definition of these units see Moore [1929].

Biological confirmation of the high vitamin A activity of some samples of colostrum. It is evident from Figs. 1 and 2 that some samples of colostrum were extremely rich in vitamin A and carotene as compared with later milk, and to leave no doubt about the results obtained by colorimetric measurements two of the richest samples were tested biologically. The following method was employed to check the colorimetric estimations. A rich sample was chosen—that yielded by the cow Foggathorpe 12 hours after calving, which had been found to contain 200 B.U. of vitamin A and 40 Y.U. of carotene in 10 g. 400 g. of this sample were weighed out and digested with concentrated potash as described above. The extraction of the fatty residue containing the carotene and vitamin A was then performed, and the residue was saponified by boiling in 25 cc. of 5 % alcoholic potash. The unsaponifiable matter was extracted and dissolved in coconut oil, sufficient of the oil being added to make 50 g. of solution. This stock solution was then examined in order to find what proportion of the colorimetrically determined carotene and vitamin A had been extracted from the colostrum and transferred to the coconut oil solution. 1 g. of the solution was weighed out, saponified and the residue dissolved in chloroform and examined as already described for carotene and vitamin A. It was found that 1 g. of the

solution contained 166 B.U. of vitamin A and 33 Y.U. of carotene, corresponding to a total of 8300 B.U. of vitamin A and 1650 Y.U. of carotene in the whole of the solution. The 400 g. of colostrum initially contained 8000 B.U. of vitamin A and 1600 Y.U. of carotene, figures identical within the limits of error of the determinations with those calculated for the whole of the stock coconut oil solution prepared, so that the colorimetrically determined carotene and vitamin A had been transferred quantitatively to the coconut oil solution.

The day doses of the stock coconut oil solution to be given in the biological test were calculated following the demonstration of Moore [1933] that the growth-promoting activities of carotene and vitamin A are approximately the same, weight for weight. The coconut oil solution contained 166 B.U. vitamin A and 33 Y.U. carotene per g., or $10/3$ B.U. vitamin A and $2/3$ Y.U. carotene per drop. (For dosing, dropping pipettes which delivered $20 \text{ mg.} \pm 1 \text{ mg.}$ of solution in each drop were used.) From the best figures available [cf. Moore and Woolf, 1932; Carr and Jewell, 1933], 1γ vitamin A = 4 B.U. and 1γ carotene = 2 Y.U., so that one drop of the coconut oil solution contained $5/6\gamma$ vitamin A + $1/3\gamma$ carotene, roughly equivalent to $7/6\gamma$ of vitamin A. The minimum dose of carotene or vitamin A required to secure growth throughout a 10-week test under conditions employed in this laboratory was shown by Moore [1933] to be about 0.5γ , therefore the dosing solution (prepared from the stock coconut oil solution weekly) was made by diluting the stock solution to $8/3$ times its own

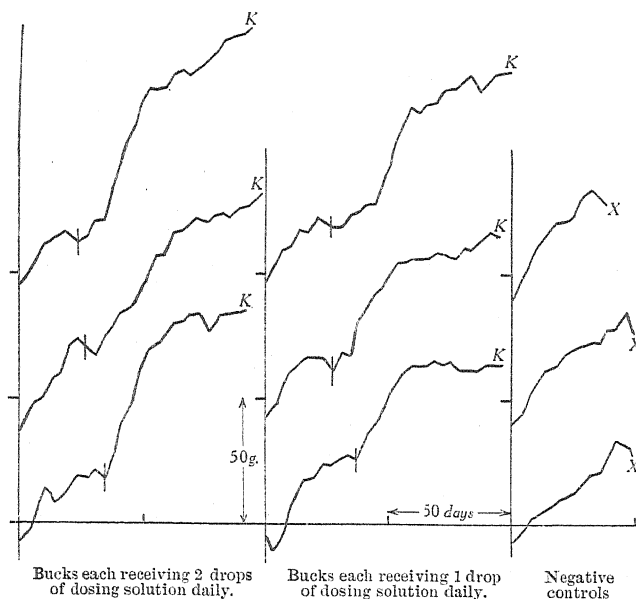


Fig. 3. Growth curves of animals used in biological test. The rats used were three trios of matched bucks. One drop of dosing solution is equivalent to 60 mg. of the second sample of colostrum obtained from the cow Foggathorpe.

weight with coconut oil, producing a dosing solution containing the equivalent of $7/16\gamma$ vitamin A per drop. Two levels were tested, 1 drop and 2 drops daily, and the weight curves of the animals appear in Fig. 3. These show that 1 drop of the dosing solution daily is barely sufficient to promote growth throughout

the 10-week period, while 2 drops daily suffice for good growth. The growth-promoting activity of the solution thus tallies closely with that to be expected from the carotene and vitamin A content estimated colorimetrically and confirms the remarkable richness of the colostrum sample. The equivalent of 120 mg. of this colostrum daily promoted rapid growth in the rat. The richness of the sample obtained from the cow Janet 3rd immediately after calving was also confirmed in the same way.

DISCUSSION.

Little previous work has been recorded on the vitamin A content of cow's colostrum. Drummond *et al.* [1921] found that "the concentration of vitamin A in the fat of colostrum is decidedly higher than in the fat of later milk," but their results give no quantitative information about their relative vitamin A contents and no other data appear to have been published.

The results tabulated above give a quantitative statement of the vitamin A and carotene content of a series of samples of colostrum from individual cows and heifers of a well controlled herd. Among the cows examined, the late milk contained generally 2-5 B.U. of vitamin A per 10 g., but many samples of colostrum contained over 100 B.U. (the highest 280 B.U.) of vitamin A per 10 g. The colostrum of every cow and heifer was richer in vitamin A than its later milk; taking a value of 3 B.U. per 10 g. as an average for the vitamin A content of the later milk of cows of this herd, then the richest samples from 4 animals contained 10-20 times, from 7 animals 30-60 times and from 3 animals 60-100 times as much vitamin A as the "standard milk." For every animal except one the colostrum had a maximum vitamin A content immediately after birth or in the next 12 hours, and the vitamin A content fell rapidly for a few days and then more slowly for a further 5-10 days until it reached the value for milk. Similarly the carotene content of early colostrum from each animal was higher than that of later milk and was very variable. The carotene and vitamin A contents of the best sample of colostrum from each animal are given in Table I in terms of the concentrations in the "standard milk."

Table I.

Maximum carotene and vitamin A concentrations of samples of colostrum of each animal in terms of the concentrations in "standard milk" of the herd. (Standard milk contains 3 B.U. vitamin A and 2 U.U. carotene in 10 g.)

Animal	Date of calving	Cow or heifer	Vitamin A concentration of richest sample of colostrum	Carotene concentration of richest sample of colostrum
Maud 6th	Dec. 1	H	70	60
Janet 2nd	Dec. 4	C	50	68
Cantab Lily	Dec. 10	H	93	53
Flora 3rd	Dec. 17	C	33	33
Star 3rd	Dec. 24	C	42	15
Lily	Feb. 6	C	10	4
Star 4th	Feb. 10	C	10	2
Russet 8th	Feb. 17	H	42	12
Constance 2nd	Feb. 27	H	42	20
Cantab Maud	Mar. 7	C	13	4
Janet 3rd	Mar. 12	H	50	7
Daisy 5th	Mar. 21	C	17	10
Foggathorpe	Mar. 25	C	66	20
Maud 4th	May 6	C	42	10
"Standard milk"			1	1

An interesting point in the carotene and vitamin A economy of the cow is revealed in Table I, which shows that although colostrum very rich in vitamin A was produced by animals calving at the end of the winter feeding period, no animal calving later than December 10th gave a colostrum very rich in carotene. Thus although the cow depends for its vitamin A on transforming the carotene of its diet [Moore, 1932], it is able to secrete vitamin A in large amounts in colostrum at a time when its diet is poor in carotene, so that it is evident that the colostrum is enriched with the vitamin at the expense of a large store in the body, presumably in the liver. A further point of interest is that the heifers generally produced richer colostrum than the cows. The average vitamin A content for 10 g. of the colostrum present at birth was: for heifers 157 B.U. (varying from 100 B.U. to 210 B.U.) and for cows 66 B.U. (varying from 10 B.U. to 150 B.U.). This distinction between the colostrum of cows and heifers is presumably due to the heifer having greater reserves of vitamin A because the reserves have not been depleted during previous lactation as with the cows. The figures for carotene content of the colostrum cannot be compared in this way because the season exerts a marked effect, and it happened that the heifers calved earlier in the season than the cows.

The importance of colostrum in supplying a reserve of vitamin A to the newborn calf is seen from the table and figures to be considerable. Kuttner and Ratner [1923] have stated that at calving the mother's udder contains from 2 to 20 lbs. of colostrum which is ingested reflexly by the calf. In view of this, the following calculation may be applied to the shorthorn cows studied. Suppose that a typical cow of the herd studied has 10 lbs. of colostrum available for the calf containing 100 B.U. per 10 g. (10 of the 14 animals gave colostrum richer than this) then the total amount of vitamin A in the colostrum available is approximately 45,000 B.U. In addition, if calving occurs during or soon after the close of the summer feeding period, there may be 45,000 Y.U. of carotene in the colostrum available at birth. Thus the calf at birth is endowed with a large reserve of vitamin A and, according to season, possibly with carotene also: a reserve which it would require weeks to build up if colostrum were no richer in vitamin A than milk.

The relative importance of colostrum in supplying vitamin A to the calf is well illustrated by figures for the cow Star 3rd. This cow gave colostrum containing 125 B.U. vitamin A per 10 g. and assuming the average quantity of 10 lbs. of colostrum, the vitamin A store available for the calf would amount to 55,000 B.U. Now this particular calf was killed by accident during birth, so that the carcass was available for examination. The liver weighed 800 g. and contained 75 B.U. of vitamin A per 100 g., or a total store of 600 B.U. The calf was born with 600 B.U. and 55,000 B.U. of vitamin were immediately available for it: this perhaps may be taken as a measure of the relative importance of the placenta and colostrum in providing the young of this species with a reserve of vitamin A until more figures can be obtained.

Further work on these lines is in progress.

SUMMARY.

1. A convenient method is described for extracting carotene and vitamin A from milk and colostrum samples.
2. This has been applied to colostrum and milk from fourteen cows of a dairy shorthorn herd.
3. The vitamin A concentration in colostrum may be from ten to one hundred times greater than in later milk from the same cow independently of season.

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4. The carotene content of colostrum may be as much as 70 times greater than in later milk unless calving occurs during late winter feeding.

5. The colostrum of heifers is generally richer in vitamin A than the colostrum of cows (on the average over twice as rich).

6. The importance of colostrum as compared with the placenta and milk as a source of vitamin A for the young calf is demonstrated. On the first day of life the calf receives supplies of vitamin A greater than the later milk could give in 20-50 days.

My warmest thanks are due to the Director of the Cambridge University Farm (Mr Mansfield) and his staff, without whose continued ungrudging assistance and interest this work could not have been contemplated.

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CCLXXII. VITAMIN C IN THE SUPRARENAL MEDULLA.

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(Received November 2nd, 1933.)

It is already known that the suprarenal gland is an extremely potent source of vitamin C, but hitherto the activity has been thought to be restricted to the cortex. In the present paper, however, it is shown that the medulla of the suprarenal is also intensely active, its potency falling but little short of that of the cortex. Our results indicate that ox suprarenal cortex has an activity of about 30 international units per gram and ox medulla of about 20 units, *i.e.* no less than two-thirds of the former. In other words the cortex has about thrice and the medulla about twice the potency of fresh orange juice or lemon juice (the international standard of vitamin C activity).

Szent-Györgyi [1928], struck by the peculiar silver-reducing power of the cortex, isolated from it the substance thought to be responsible—first called hexuronic acid, but later, after its identification with vitamin C, renamed ascorbic acid. The intense antiscorbutic activity of the suprarenal cortex was demonstrated in papers from this laboratory [Harris, Mills and Innes, 1932; Harris and Ray, 1932; 1933, 1] and the degree of activity was shown to be commensurate with its richness in hexuronic acid. A chemical method for estimating the latter, based on modifications in the use of Tillmans's oxidation-reduction indicator 2: 6-dichlorophenolindophenol was worked out and applied to various animal and vegetable materials, and the surprising discovery was made that certain tissues, including the suprarenal medulla, not hitherto recognised as sources of vitamin C, also gave a high titration value [Harris and Ray, 1933, 1, 2; Birch, Harris and Ray, 1933; Birch and Dann, 1933; Harris, 1933, 1]. This result at once suggested that the absence of the silver reaction was in reality little guide as to the presence or absence of vitamin C, a conviction which was strengthened by our further observation that the medulla and cortex of different species, as well as a variety of other tissues (*e.g.* liver), might stain or not in the most erratic order with little apparent parallelism with their true antiscorbutic activities. Again liver extract and other tissue extracts although rich in vitamin C did not reduce silver. Similarly Gough and Zilva [1933] have recently noted that human suprarenals may fail to darken at all with silver (their vitamin C potency, however, not having been tested). The chemical test for vitamin C, although it is known to be reasonably specific, may give high results in certain exceptional cases, and it was therefore necessary to check the titration results on the suprarenal medulla against direct feeding tests. The biological determination gave results in complete agreement with the indophenol titration. We have recently repeated these feeding tests and exactly confirmed our earlier results.

EXPERIMENTAL.

The technique, using the histological tooth structure method, was the same as that described in earlier papers [*e.g.* Harris and Ray, 1932].

In the first test a group of three guinea-pigs received daily 1 g. of freshly excised ox suprarenal medulla and control groups received 1, 2 and 3 cc. of orange juice. The second test was similar except that the last group was omitted. The results are shown in Table I, and are analysed in Fig. 1.

Table I. *Antiscorbutic activity of ox suprarenal medulla.*

Supplement	Degree of protection	
	Individual animals	Mean
First determination		
Suprarenal medulla, 1 g.	2, 2, 3	2.3
Negative control	0, 0.5, 1	0.5
Orange juice, 1 cc.	1, 2, 1, 1.5	1.4
2 cc.	3, 2.5, 2.5, 2	2.5
3 cc.	3.5, 3.0, 1.5, 3.5	2.9
Second determination		
Suprarenal medulla, 1 g.	1.5, 1.5, 2	1.7
Negative control	0, 0, 0	0
Orange juice, 1 cc.	0.5, 1, 1.5	1
2 cc.	1, 2, 2.5	1.8

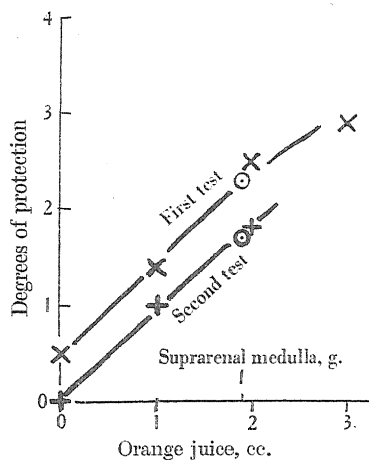


Fig. 1. Comparison of protective power of suprarenal medulla and orange juice.

x orange juice; o medulla.

Results. As is seen from Fig. 1, 1 g. of suprarenal cortex was found to have the same biological activity as 1.9 cc. of orange juice. This same figure was obtained in both series of tests. It agrees exactly also with that calculated from the chemical titration, according to which 1 cc. of orange juice was found to contain 0.6 mg. ascorbic acid, and 1 g. of medulla 1.1 mg. (see Table II).

Lack of correlation with silver stain. It is seen that although the ox medulla does not stain with silver in Szent-Györgyi's method, and the cortex does, nevertheless the medulla contains vitamin C in almost the same order of

Table II. *Vitamin C content of ox suprarenal medulla: agreement between biological and chemical results.*

	Amount of orange juice equivalent to 1 g. of medulla
By biological test	1.9 cc.
By chemical test	1.8 cc.

concentration as does the cortex. It is apparent at once, therefore, that the absence of a silver stain is no infallible indication that large amounts of vitamin C may not be present in a given tissue. Liver and certain other tissues appear in fact to contain a substance or system which protects silver nitrate from reduction by ascorbic acid. Thus we find that when ascorbic acid is mixed with the tissue extract it no longer reduces silver.

PHYSIOLOGICAL CONSIDERATIONS.

1. *The suprarenal not a reserve store of vitamin C.* At first sight the fact that the suprarenal contains a concentration of vitamin C so many times greater than that present in the body as a whole might be thought to indicate that it serves as a reserve or storehouse, in the same way that the liver is known to put away reserves of vitamin A for the future use of the body. This interpretation indeed seems to have been already adopted in some quarters. The following considerations, however, show that this conception cannot be accepted. In the first place, the total amount of ascorbic acid in the adrenals of the guinea-pig is no more than about 0.5 mg.; *i.e.* not sufficient to provide the vitamin C needs of the body for more than about 24 hours. This should be contrasted with vitamin A, of which enough can easily be held in the liver to provide an animal with its needs for a whole lifetime. Secondly, we find that the amount in the suprarenal does not appear to be raised significantly above normal limits after extra allowances of vitamin C have been given. This finding again is in sharp contrast with all experience with vitamin A, where the reserves, and hence the time of depletion, depend entirely on the pre-experimental diet. Thirdly, if the vitamin were held as a reserve we should expect it to disappear from the suprarenal during the early stages of a vitamin C-free regimen and no scorbutic symptoms to develop until such reserves had been drawn upon. This is what happens with vitamin A, no ill-effects beginning to appear until the very last traces of vitamin A have been used up from the liver. But with vitamin C on the contrary we find that the amount in the suprarenal diminishes gradually during the whole course of scurvy. At the tenth or twelfth day when teeth lesions are already far advanced there may still be considerable amounts of ascorbic acid in the suprarenal; and again, at death from scurvy it is not unusual to find small amounts of ascorbic acid still present in the organ.

2. *The suprarenal and the synthesis of vitamin C by certain species.* In an earlier paper [Harris and Ray, 1933, 2] we mentioned that the suprarenal of the rat was considerably richer in vitamin C even than that of the ox or normal guinea-pig, judging from the titration result, the activity being ten times greater than that of orange juice, or 6 mg. of ascorbic acid per g. Biological tests (curative method) have confirmed this conclusion, both for normal rats' suprarenals and for the suprarenals of rats which had been kept for long periods on a vitamin C-free diet (Fig. 2).

Since the rat is known to synthesise its own vitamin C (as the above test further demonstrates), and one of us has shown the same to be true also of the dog [Harris, 1931], it seemed a possibility that the suprarenal might be the site

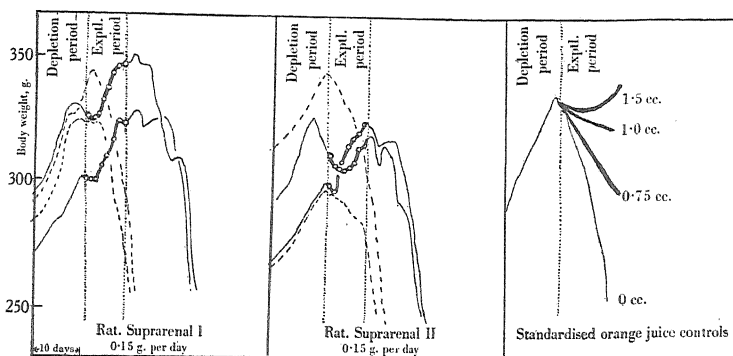


Fig. 2. Curative tests on guinea-pigs, with rat suprarenals from rats (I) fed on vitamin C-free diet and (II) fed on the vitamin C-free diet *plus* cabbage.

— experimental curative period; — vitamin C-free diet; — — — negative controls.

of the synthesis in such species. In order to try and test this working hypothesis, a number of rats were submitted to adrenalectomy¹ and then placed on a vitamin C-deficient diet, consisting of

Bran	80 parts by weight
Oats	720 "
Egg-yolk	40 "
Salts	8.4 "
Cod-liver oil	1 %

(Provision of eucortone or adrenaline appears to be unnecessary for this species.) If the suprarenal were in fact concerned in the synthesis of vitamin C in the rat, we had hoped that symptoms of vitamin C deficiency might appear. However, the rats seemed to thrive normally after the operation and on the vitamin C-free diet. Unfortunately this finding can only be regarded as a negative result and cannot be taken as final and conclusive evidence of lack of suprarenal synthesis: since two objections might legitimately be raised—first, that small amounts of accessory tissue had escaped removal; and, secondly, that there had occurred some regeneration of new tissue after the operation. We did in fact observe in three out of four animals killed after four weeks what appeared to be rudiments of such new tissue. Taking all considerations into mind, however, the weight of evidence seems to be definitely opposed to the theory that the synthesis of vitamin C, which is known to occur in species like the dog or rat, has its seat in the suprarenal.

If, as appears likely, the physiological properties of ascorbic acid depend essentially on its specialised reducing properties, it may be supposed that the large amounts in the suprarenal cortex are involved in a system needed to maintain adrenaline-like substances in a reduced condition. Work in another connection [Harris and Fish, 1928–29; Harris, 1933, 2] has shown that in certain other sites vitamin C seems to be needed primarily for maintaining the functional

¹ We are indebted to Prof. J. H. Burn for kindly demonstrating the technique.

activity of certain types of cells and preventing their premature degeneration, e.g. osteoblasts, odontoblasts, ameloblasts, cementoblasts (so that vitamin C in this respect may be contrasted with vitamin D which does not act directly on cell structure in the bone and teeth but through phosphate and calcium metabolism). In tissues such as the tooth pulp and growing bone, however, in contrast with the suprarenal (and certain other organs) in which vitamin C appears to play an equally important rôle, it may be noted that there is no appreciable increased local concentration of vitamin C.

SUMMARY.

1. The medulla as well as the cortex of the suprarenal is intensely rich in vitamin C.
2. The cortex (in the ox) is about thrice and the medulla about twice as potent as orange juice (or standard lemon juice), weight for weight.
3. The biological results agree precisely with the chemical titration, showing that ox suprarenal medulla contains about 1.1 to 1.2 mg. of ascorbic acid per g.
4. A negative silver stain is no certain guide as to the presence or absence of vitamin C.
5. The significance of the localised concentration of vitamin C in the suprarenals is discussed. It is shown that it cannot be regarded as a reserve store for the body, comparable for example with the vitamin A resources of the liver. It is thought more probable that it is needed for protecting the normal functional activities of the organ. To test the theory that the suprarenal is engaged in synthesising the vitamin in such species as are known to make their own (such as dogs and rats), adrenalectomised rats were fed on vitamin C-free diet for periods of several weeks to see whether scurvy developed, but with negative results.
6. Rat suprarenal has the extremely high activity of about three times that of the ox or normal guinea-pig, or ten times that of orange juice, as determined biologically and by titration (6 mg. ascorbic acid per g.).

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CCLXXIII. THE EXCRETION OF VITAMIN C IN HUMAN URINE AND ITS DEPENDENCE ON THE DIETARY INTAKE.

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(Received November 2nd, 1933.)

PREVIOUS papers from this laboratory have dealt with the application of the titration method with 2:6-dichlorophenolindophenol to the estimation of vitamin C in various animal materials [Harris and Ray, 1933, 1, 2; Birch *et al.*, 1933; Birch and Dann, 1933; Harris, 1933]. Results on human urines are discussed in the present communication. A principal purpose we had in mind in undertaking this enquiry was to discover whether any relation could be found between urinary loss and the state of nutrition of the individual in respect to vitamin C sufficiency or subnormality. Any such index would obviously be of great value for practical human dietetics.

Previous biological work has failed to demonstrate the presence of vitamin C in urine [van der Walle, 1922], but a note just published by Eekelen *et al.* [1933] mentions, without giving actual data, that the reducing substance in urine is higher in persons using much fruit.

METHOD.

The micro-method of Birch *et al.* [1933] was used throughout. The urine to be examined was made acid with trichloroacetic acid (final concentration of latter, 5 %) and titrated from a micro-burette reading to 0.01 cc. against 0.05 cc. of the indicator, which had been previously standardised against ascorbic acid. Titrations were carried out immediately after, or within a few minutes of, urination, as we found that the titre tended to fall in urine which had been allowed to stand for long. It is essential to carry out the titration rapidly and reach the end-point within about 1 min., otherwise erroneously high values will be caused by phenolic or similar substances in the urine reducing the indicator slowly. Urines are sometimes encountered which are too dilute to give a satisfactory end-point. If a reading is required for such an individual it is necessary to restrict his fluid intake.

The results to be described make it clear that the titration figure bears a genuine relation to vitamin C metabolism, but in expressing our results for convenience in terms of so many mg. of ascorbic acid we wish to make it clear that it is without prejudice to the question of the invariable specificity or otherwise of the reaction.

EXPERIMENTAL.

Excretion after large dose. Our most detailed observations were undertaken with the object of obtaining a quantitative picture of the course of excretion of the vitamin after the administration of a single large dose. These results will be

described first. In the experiment represented in Fig. 1, A. W. drank 600 cc. of orange juice at 9 a.m., restricted himself for several days thereafter to a vitamin

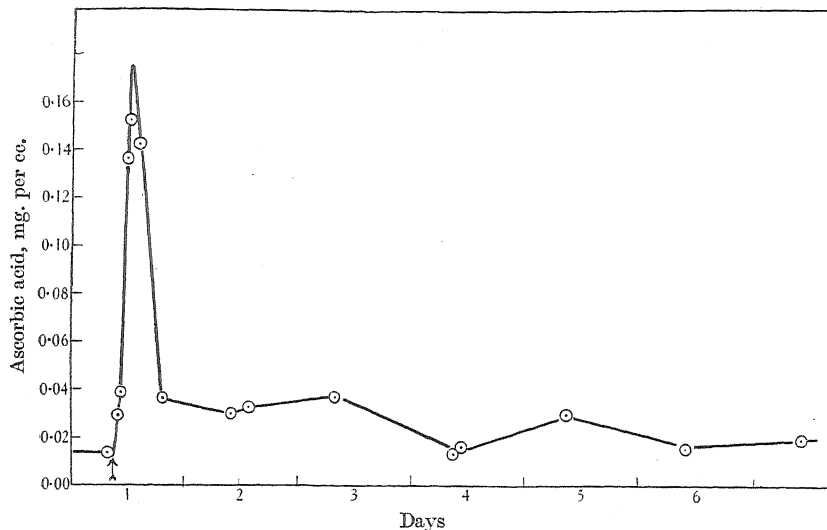


Fig. 1. Sudden rise in concentration of vitamin C in urine following large dose of orange juice (given at arrow).

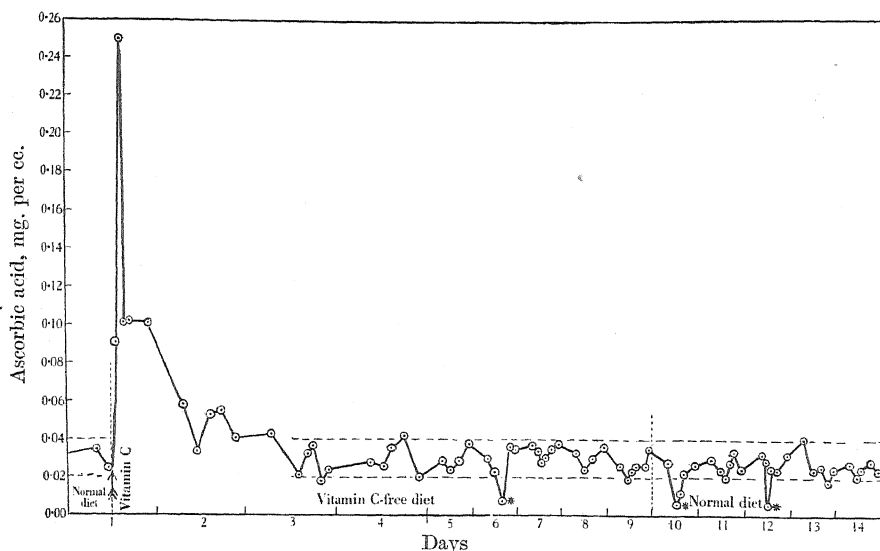


Fig. 2. Complete 14-day (half scale) record of vitamin C concentration in urine. Sudden rise in concentration after large dose of orange juice (given at arrow), followed by fall to within normal limits within 48 hours. * = diuresis after beer drinking.

C-free diet, and estimated the vitamin C content of his urine at frequent intervals. The results show that the vitamin C excretion had already begun to rise markedly after 1 hour, had reached a maximum in about 3 hours, and fallen to the original level again by the end of the third day. At the third hour, at the peak of the

curve, the concentration of vitamin C in the urine was no less than seven times greater than the normal figure. In nine hours, 76 mg. out of the original 300 mg. taken (the latter value being based on the titration figure) had been voided, and in the course of 3 days about 250 mg.

A second experiment was conducted along similar lines (Figs. 2 and 3), and the same typical sudden rise followed by rapid fall to a level value was again seen. In this experiment 556 mg. of ascorbic acid were taken in one dose

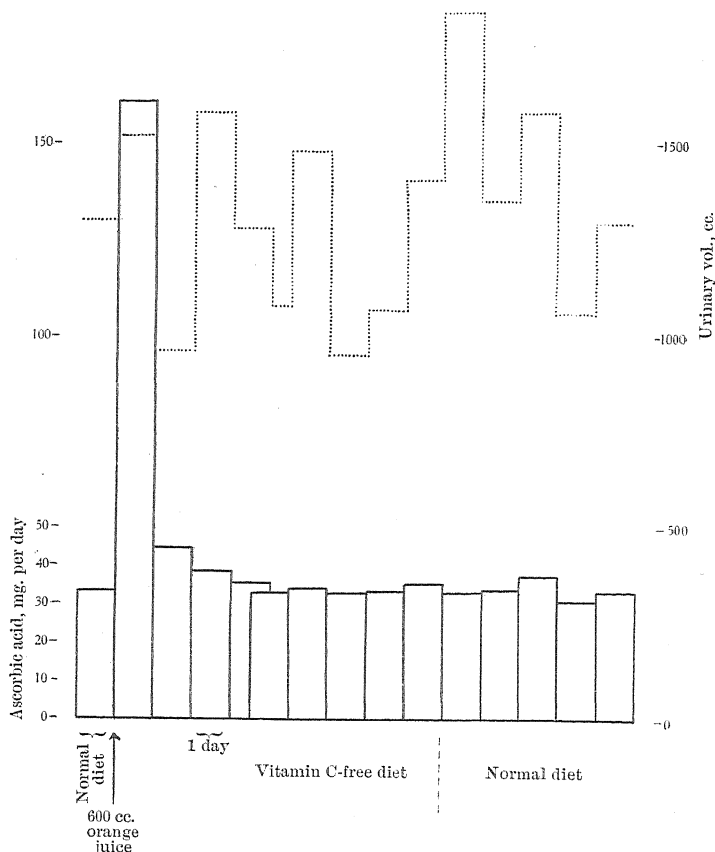


Fig. 3. Total daily outputs of vitamin C in urine, showing increase after large dose, and, later, steady output of about 33 mg. per day.

(600 cc. orange juice) and observations were continued for over a fortnight afterwards. It will be noted that the maximum rate of excretion was reached in 3 hours, that after 24 hours the excretion was only little above normal, and that it had quite dropped back to normal after 50 hours. (At the peak the urine contained as much as eight or ten times the normal concentration of vitamin C and the total weight of ascorbic acid excreted in the first 10 hours was 132.4 mg. compared with a normal of about 20 mg. for a corresponding day-time period.) For a further 6½ days the vitamin C-free regimen (eggs, cheese, butter, bread, various cereals) was continued, and it was found that the amount of vitamin C excreted continued to remain extremely constant, between limits of

32.5 and 35.3 mg. per day (average 33.6), and with an average concentration of about 0.03 mg. per cc., a level we find to be characteristic of most of the normal specimens we have examined.

No departure from this steady state occurred when the subject recommenced a normal diet, 10th to 15th day of experiment.

Normal urines. Tests on urines from normal individuals on ordinary mixed diets are recorded in Table I. All are males. The first four are laboratory

Table I. *Urinary excretion of vitamin C by men on normal diets.*

Subject	Time	Total volume of urine passed cc.	Weight of vitamin C in total specimen mg.	Concentration of vitamin C in urine mg. per cc.
G. G. G.	10.30 a.m.	165	8.35	0.050
	2.45 p.m.	130	6.96	0.053
	9 a.m.	610	12.85	0.021
				Av. 0.032
S. G. I.	12 noon	140	2.71	0.019
	1 p.m.	75	1.45	0.019
	4.30 p.m.	255	7.26	0.028
	9 a.m.	360	10.11	0.028
	1 p.m.	235	4.77	0.020
				Av. 0.024
A. W. D.	10.30 a.m.	230	10.44	0.045
	1 p.m.	115	5.03	0.044
	4.30 p.m.	160	2.55	0.016
	5.30 p.m.	110	4.64	0.042
	9 a.m.	725	15.47	0.021
				Av. 0.028
S. A. C.*	10.30 a.m.	115	—	<0.014
	1 p.m.	210		
	2.45 p.m.	305		
	4 p.m.	260		
	5 p.m.	260		
	9 a.m.	740		
	1 p.m.	500		

* Diuretic specimen.

assistants. It will be seen that the figure is generally very close to 0.03 mg. per cc. of urine, and the daily output does not vary far from about 30 mg. (see Table II). An example is included of one subject (S. A. C.) who had diuretic tendencies, and the concentration was insufficient to permit a clear end-point being reached in the titration.

Table II. *Average concentration and daily output of vitamin C in urine in normal males.*

Subject	mg. of vitamin C per cc. of urine	Approximate total output (calculated) (mg.)
A. W. D.	0.028	38.1 in 24 hours
G. G. G.	0.032	28.2 "
S. A. C.*	<0.01	—
S. G. I.	0.024	ca. 10.0 in 10-12 hours
S. N. R.	0.027†	—
T. W. B.	0.022†	—
W. J. D.	0.017†	—
L. J. H.	0.016†	—

* Diuretic specimen.

† Based on single titrations only.

Results quoted in the last section show also that the urine which has collected in the bladder during the night and is excreted first thing in the morning often tends to become rather more concentrated as regards the vitamin.

Since the daily output of vitamin C in the urine seems to be at so constant a level in many individuals it is of interest to compare it with the reputed minimum daily need. This is stated to be "1 oz. of orange or lemon juice per day" (*i.e.* about 20 mg. of ascorbic acid). It would appear therefore that the average individual excretes daily in his urine an amount of vitamin C of a similar order to, but definitely somewhat greater than, the bare amount needed to prevent scurvy. It may be expected from this that whenever the vitamin C intake falls below a safe margin an effect should be readily apparent on the urine. We hope to present data of urinary excretion in Barlow's disease and the response after vitamin C as compared with normals in illustration of this. The most accurate general procedure would appear to be to determine the total daily output after 48 hours on a vitamin C-free regimen. This rules out irregularities due to abrupt changes in urinary volume and to the more immediate effects which follow directly on the administration of a single dose of the vitamin; if necessary also the fluid intake may be restricted in order to give a urine concentrated enough to ensure a sharp end-point in the titration.

SUMMARY.

1. The vitamin C content of human urines has been determined by the titration method.
2. In a number of normal individuals the amount of vitamin C excreted in the urine was surprisingly constant, generally in the neighbourhood of 30 or 33 mg. per day, or roughly 0.02–0.03 mg. per cc. of urine (this concentration being naturally lowered in diuresis, and being sometimes rather higher in the more concentrated early morning urine).
3. If a normal individual is given a single very large dose of vitamin C (*e.g.* 600 cc. of orange juice, 30 times the reputed daily requirement), the concentration of vitamin C in the urine sharply rises, reaching in about 3 hours a maximum as high as, *e.g.*, 8 or 10 times the "normal"; it then rapidly drops again and within a day or so reaches the "normal" resting level of about 33 mg. excreted per day where it remains remarkably steady although the subject may be restricted, *e.g.* for a week or more, to a vitamin C-free diet.
4. This "normal" daily loss of about 33 mg. is somewhat greater than the reputed minimum daily requirement for man (*viz.* "1 oz. of orange or lemon juice").
5. The technique described has possible applications for dietetics, for the diagnosis of hypovitaminosis-C in human beings.

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CCLXXIV. STANDARDISATION OF THE ANTI-SCORBUTIC POTENCY OF ASCORBIC ACID.

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(Received November 2nd, 1933.)

THE results to be described in the present paper confirm conclusions given in an earlier note [Harris and Ray, 1933]. We reported there that ascorbic acid—specimens derived from two different raw materials were examined—had an activity such that 2 mg. were equivalent to 3 cc. of orange juice. This would appear to indicate an activity of 15 International Units per mg., since orange juice is generally assumed to have an activity about equal to that of lemon juice, and the ascorbic acid contents as estimated by titration are similar. To confirm the original finding, and obtain a value of as highly precise a nature as possible a rather more elaborate test was then carried through and concluded in May 1933, and the same result was again obtained. A note by Key and Morgan [1933] which has just appeared gives a somewhat lower value than ours—*viz.* 7.4 instead of 15. Our results, although they are based on the use of orange juice as a secondary standard and not on a direct comparison with lemon juice as primary standard (which, however, may vary considerably in potency, as shown below), have the advantage of being founded on more extensive statistical data and involve also a more direct comparison with standard material fed simultaneously at the identical levels of potency, and we think it may be of interest to publish the details.

EXPERIMENTAL.

The ascorbic acid was a very pure specimen from paprika, several times recrystallised and giving an iodine titration of over 99 % of the theoretical. The tooth structure method [Höjer, 1924; 1926; Key and Elphick, 1931] was used, the general procedure being the same as previously indicated [Harris and Ray, 1932]. Ascorbic acid was fed to groups of guinea-pigs at three different levels 1.5 mg., 2.0 mg. and 2.5 mg. per day. (The doses were weighed out daily and dissolved in air-free water immediately before administration, the dilution being such that 1 mg. of ascorbic acid was present in 2 cc. of water. The main specimen was kept in an atmosphere of CO₂ and stored dry at 0°.) Simultaneously three different levels of orange juice were fed to controls, at what were adjudged, on the basis of the earlier tests, to be corresponding levels of activity, *viz.* 2.25 cc., 3 cc. and 3.75 cc. Six animals were included in each group. These particular levels of activity were chosen as giving a fairly full degree of protection, a condition which we find to give more consistently reproducible and sensitive results than at lower partial levels of protection. The animals were killed after 15 days and cross-sections of the incisors prepared in the usual way and assessed for degree of protection.

Our use of orange juice as a secondary or derived standard in place of lemon juice used direct as primary standard may be criticised, but we believe it to have some advantages. Previous papers both from the Pharmaceutical Laboratory and this laboratory have recorded that more constant and easily reproducible results were obtained with orange juice than with lemon juice, apart of course from the additional consideration that the animals take it better, and there is no need to introduce a decitration process when feeding the larger doses which are needed at top levels in the tooth protection method. Confirmation has recently been forthcoming in the report of Bennett and Tarbert [1933] that lemon juice may indeed vary most erratically in potency, the vitamin C content as measured by titration in poorer specimens being little more than about half that of a "good" specimen, whereas orange juice tended to be more constant with a value about equal to or very slightly above that of a "good" lemon juice. It is obvious therefore that the result obtained in any vitamin C determination will depend on the interpretation attached to the standard "lemon juice," whether this is taken to imply lemon juice of a "good" quality, of "low," or of "average" quality (if such can be defined). Key and Morgan have also referred to the apparent variation in potency of lemon juice.

RESULTS.

The values found are given in Fig. 1 and for the third determination in Table I. When these are plotted it is seen that the readings for orange juice and ascorbic acid are equated when the scales for orange juice and ascorbic acid respectively are fixed at 3 cc. of the former against 2 mg. of the latter. This conforms with the earlier findings. It coincides, also, with the amount of

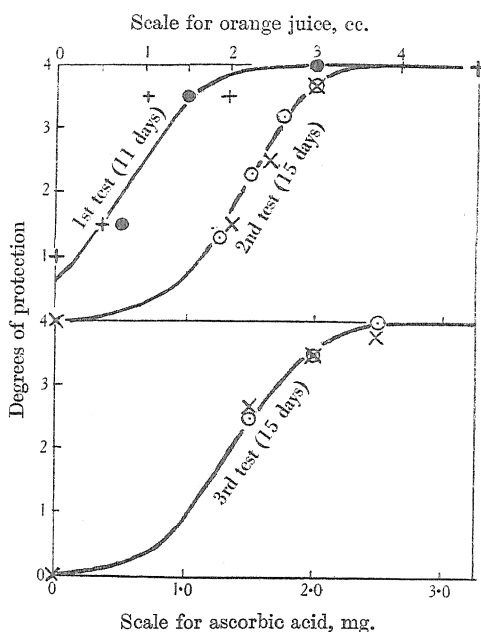


Fig. 1.

○ ascorbic acid; × orange juice.

Table I.

Amount of material fed per day	Degree of protection afforded							Mean
	Individual animals							
Ascorbic acid, 1.5 mg.	2.5,	2,	2.5,	2,	3,	—		2.5
2 mg.	4,	3,	3.5,	3,	4,	3.5		3.5
2.5 mg.	4,	4,	4,	4,	4,	—		4
Orange juice, 2.25 cc.	2,	4,	2,	3,	2.5,	—		2.7
3 cc.	2.5,	4,	4,	3,	3.5,	4		3.5
3.75 cc.	4,	4,	4,	3.5,	3.5,	—		3.8

ascorbic acid in an average specimen of orange juice as found by titration in our experience (*viz.* 0.6 to 0.7 mg. per cc.). It represents a value of about double Key and Morgan's.

COMMENTS.

In Key and Morgan's determination, one level only of the lemon juice standard was fed in each experiment and that was administered at levels widely divergent from those of the "unknown" (especially in Exps. 1 and 2). In order to bridge the gap recourse was had to a standard reference curve relating degree of protection in any experiment to the relative amount of vitamin which is needed to produce it. Although we feel that it is in fact a safer procedure to obtain several points on the curve for each determination, and to have one point, at least, for the unknown always lying between two reasonably adjacent values for the standard (or *vice versa*), it nevertheless seems likely that the difference between our result and that of Key and Morgan is mainly attributable to another cause—*viz.* the relatively high rating given by them to their single lemon juice standards. There is quite good agreement between Key and Morgan's values and our own as to the actual degrees of protection afforded by the varying doses of ascorbic acid. Their results agree with ours in showing that 2 mg. are about the minimum dose for giving approximately full tooth protection in a 15-day test. Turning now, however, to Key and Morgan's readings for lemon juice, it would appear that the potency was assessed at double the value that might have been anticipated. Thus, 1.25 cc. of lemon juice gave the same protection as 2.30 cc. of the orange juice of the standard reference curve, and 1.0 cc. of lemon juice as 2.21 cc. of orange juice; and their combined results [Fig. 2, Key and Morgan, 1933] would imply that full protection would be given by as little as 1.48 cc. of lemon juice. This may be compared with the more usually accepted finding that about 3.0 cc. (*i.e.* double the amount) of orange or lemon juice are in fact normally needed to give full protection. One explanation of the apparent divergence between their results and ours, then, would be that they were dealing with a lemon juice of exceptionally high potency, or alternatively that our orange juice was relatively low; or both. This is not necessarily the correct explanation however; indeed orange juice tends on the whole to be somewhat more potent and not less potent than lemon juice. It could be equally well argued that the great variation between the individual animals in Key and Morgan's small number of lemon juice groups could have given rise to an illusively high average. Thus with 1.25 cc. of lemon juice the individual scores ranged from 1.5 to 4.0 and the average was put as 3.2; with 1.0 cc. of lemon juice they ranged from 0.5 to 4.0, with average at 3.1. If there had been a variation in 1 or 2 animals, within the actual range found in the group as a whole, it could easily have reduced the average score to the anticipated value. (In the case of

ANTISCORBUTIC POTENCY OF ASCORBIC ACID 2019

Table II. *Ascorbic acid content of lemon juice and orange juice.*

I. Lemon juice			
Day	Specimen number and description	Ascorbic acid content as determined by titration mg. per cc.	Corresponding calculated activity of ascorbic acid units per mg.
1st	1 (small)	0.515	19.4
	2 (large)	0.407	24.6
	3 (small)	0.583	17.2
	4 (very large)	0.183	53.2
	5 (medium large)	0.380	26.3
	6 (large)	0.44	22.7
	7 (large)	0.41	24.4
	8	0.475	21.1
	9	0.506	19.8
	1st day, average of 9 specimens	0.43	23.3
2nd	10	0.536	18.0
	11 (small and withered)	0.684	14.6
	12 (large and fresh)	0.354	28.3
	13 (small and withered)	0.661	15.1
	14 (very small and withered)	0.694	14.4
	2nd day, average of 5 specimens	0.59	17.0
3rd	15	0.373	26.8
	16	0.381	26.3
	17	0.669	15.0
	18	0.339	29.5
	19	0.472	21.2
	20	0.342	29.2
	3rd day, average of 6 specimens	0.43	23.3
	Three days, average of 20 specimens	0.47	21.3
4th	A composite specimen = mixed juice of 10 lemons	0.47	21.3
II. Orange juices			
1	1 (South African)	0.570	17.5
	2 "	0.570	17.5
	3 (Brazilian)	0.480	20.8
	4 "	0.620	16.1
2	5 (South African)	0.806	12.4
	6 "	0.909	11.0
3	7 "	0.628	15.9
	8 "	0.833	12.0
	9 (Brazilian)	0.757	13.2
	10 "	0.585	17.1
4	11 "	0.586	17.1
	12 "	0.510	19.6
5	13 "	0.533	18.8
	14 "	0.476	21.0
	Average of 14	0.63	15.8

Table III. *Ascorbic acid content of orange juice.*

As determined by titration	...	0.63 mg. per cc.
As calculated from biological assay	...	0.66 "

the first experiment, with 0.75 cc. of lemon juice and 0.3 mg. of ascorbic acid, there is difficulty in extracting a relationship, since the degree of protection was only very partial in both cases and the lemon juice was being fed at over 3 times the level of potency of the ascorbic acid, making comparison very insecure¹.)

In conclusion we would allude again to the fact that our value coincides with the actual ascorbic acid content of average orange juice or good lemon juice, as determined by chemical titration. The same correlation holds equally for a number of other sources [Harris and Ray, 1933]. This seems a strong argument in its favour. Key and Morgan's value of 7.4 units per mg. would indicate an ascorbic acid content of 1.35 mg. per cc. for lemon juice, a value which would appear difficult to reconcile with the fact that in our experience the reducing titre rarely rises above a value equivalent to about 0.7 mg.

Titration results on a series of lemons and oranges purchased locally from retailers in October are given in Table II. The average ascorbic acid content of the lemon juice (based on determinations on 20 separate lemons, and on the mixed juice of a further 10) was appreciably lower than that of the oranges examined at the same time (*viz.* 0.47 mg. per cc. for lemon juice, as against 0.63 for orange). It must be noted, however, that individual lemons show striking fluctuations in potency, the mean deviation from the average value of 0.47 mg. per cc. being as great as ± 0.11 . (It was observed that higher values were generally given by lemons which were small and shrunken in appearance, and lower values by large "juicy" ones—as though the juice became more concentrated by evaporation during the storage process.) The figure for the orange juice is in agreement with our biological determination of the relative activities of ascorbic acid and orange juice (Table III) although we have found that different varieties of orange juice may also vary considerably in potency. If lemon juice of the "average" quality represented by these specimens is taken as standard, the calculated antiscorbutic activity of ascorbic acid works out at the somewhat higher value of 21 units per mg., as against the value 15 units per mg. compared with a "good" lemon juice equal in potency to that of the orange juice used in our tests.

Since "lemon juice" without further specification has already been fixed as International Standard, and in our opinion the antiscorbutic activity of lemon is equivalent to its ascorbic acid content as estimated by titration, the final step in transferring the standard from lemon juice to ascorbic acid will have to involve a decision as to the exact quality of lemon juice which may be held to represent a fair and normal specimen.

SUMMARY.

1. Assays by the tooth protection method, using large numbers of guinea-pigs, and with ascorbic acid and orange juice always fed simultaneously at various identical levels of potency, show consistently that the activity is such that 2 mg. of ascorbic acid are equivalent to 3 cc. of orange juice, or the minimum dose for full tooth protection (under the conditions of the test) is 2 mg.

¹ In our experience more precise results are obtained by comparing the minimum doses needed to produce complete protection in a given large proportion of animals, rather than by comparing the doses needed to produce a partial (and generally rather variable) degree of protection. Another source of error is that a slight change in the level at which the cross-section of the tooth is cut greatly affects the picture. The guinea-pig's tooth is of persistent growth and the upper part (away from the root) is that formed before the experimental period, so that if the section be cut high enough it will appear quite normal. More accurate conclusions are formed from longitudinal sections.

2. This value agrees with the amount of ascorbic acid actually present in average specimens of orange juice or in "good" lemon juice, as determined by titration (0.6–0.7 mg. per cc.).

3. Lemon juice is subject to some variation in potency, so that the evaluation of ascorbic acid in terms of International Units will depend on the quality of the lemon juice used for comparison, but taking the interpretation that the standard lemon juice means a "good" specimen, equivalent to the orange juice used in our test and showing 0.6 to 0.7 mg. of ascorbic acid per cc. on titration, ascorbic acid has a potency of 15 International Units per mg. Against "average" lemon juice as purchased retail in Cambridge, having an ascorbic acid content of 0.47 mg. per cc., the calculated activity for ascorbic acid works out rather higher, *viz.* 21 International Units per mg.

4. The alternative value 7.4 units per mg. put forward by Key and Morgan would indicate an ascorbic acid content of 1.35 mg. per cc. for lemon juice, which seems difficult to reconcile with the fact that the reducing titre rarely rises above a value equivalent to about 0.7 mg. Possible reasons for the lack of agreement are discussed.

5. Our earlier conclusion is confirmed, that lemon juice is an unsatisfactory standard and should be replaced by ascorbic acid. Different specimens of lemon juice had ascorbic acid contents varying from 0.19 to 0.69 mg. per cc., average 0.47 with an average deviation of ± 0.11 .

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CCLXXV. THE ENDOCRINE FACTORS CONCERNED IN THE CONTROL OF THE OVARIAN CYCLE.

II. *RANA TEMPORARIA* AS TEST ANIMAL.

III. THE ACTION OF ANTERIOR LOBE PITUITARY EXTRACTS ON THE OVARY.

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(Received October 10th, 1933.)

II. *RANA TEMPORARIA* AS TEST ANIMAL.

It is now generally believed that the nature and extent of the periodic changes occurring in the ovary are controlled by the anterior lobe of the pituitary. The study of this relationship has been seriously handicapped, however, because the gonad-stimulating properties of extracts and other preparations can only be adequately studied in the hypophysectomised animal. Since it is practically impossible to remove the anterior lobe of the mammal alone or even carry out total hypophysectomy with ease, progress is beset by serious obstacles. In a previous paper [Bellerby, 1933] it was pointed out that this difficulty can be overcome by making use of certain amphibia, and attention was drawn to the suitability of the African Clawed Toad (*Xenopus laevis*) for use in the study of the ovary-pituitary relationship particularly when used for the assay of a gonad-stimulating substance. Before the practical possibilities of *Xenopus* were ascertained experiments were carried out to determine the suitability of the common English frog (*Rana temporaria*) for the same purpose.

As regards the removal of the pituitary *Rana* possesses the same main advantages as *X. laevis* [Hogben, 1923].

- (a) It can be easily and rapidly hypophysectomised without injury.
- (b) One or both lobes of the pituitary can be removed with precision.
- (c) Completeness of removal can be checked by the characteristic colour response of the animal.

The present investigation shows that *Rana* is much less satisfactory than *Xenopus* for use in the study of the gonad-stimulating properties of extracts. It can nevertheless be used with advantage to confirm conclusions derived from experiments on *Xenopus* and will be made use of in the present studies for that purpose.

EXPERIMENTAL.

After the female has spawned in the spring the resultant atrophic ovary undergoes rapid development throughout the year until it is practically fully developed again by the autumn. A period of slow growth then occurs until the following March or April. The ovary has finally increased about ten times in weight and consists almost exclusively of black ova.

At spawning time the ova are first liberated *en masse* into the body cavity (ovulation *sensu stricto*). Then as a result of muscular movements of unknown origin they enter the oviducts which become enormously distended, leaving the ovary yellowish in colour and a mere vestige of its former size. The ova are finally expelled from the oviducts through the cloaca (oviposition) as a result of some stimulus the nature of which is at present unknown. The process takes place in three distinct stages, and some time may elapse between ovulation and oviposition.

In the present series of investigations suspensions of anterior lobe tissue were employed. The sole reason for this is that experiments were carried out before the work on *Xenopus* was undertaken. It was not until some progress had been made with the latter that it was found that extracts yielded consistent results.

Suspensions were prepared as follows. The glands were dissected out within 4 hours of the death of the animal and were ground to a fine paste with sand. The mass was agitated with twice its weight of normal saline for about 10 minutes and centrifuged. The fine suspension was then decanted off the sand and residual tissue and injected without delay. All injections were made into the dorsal lymph sac.

The great advantage of *X. laevis* as a test animal is that eggs are ejected externally within 24 hours as a result of a single injection of an active preparation. This dispenses with the necessity of killing the animal. An attempt was therefore made to procure a similar reaction in *Rana*. Two series of experiments were carried out.

In the first, frogs were injected with a volume of suspension equivalent to 2 g. of original tissue and were killed 3 days later. Oviposition only occurred in 2 cases, one frog ejecting three ova on the 2nd day, the other two ova on the 3rd day. The result therefore was quite abnormal because in the normal frog several hundreds of ova are shed at one time.

In Table I are given details of the *post mortem* results. It was somewhat surprising to find that although definite oviposition had not occurred marked

Table I.
Weights in g.

Injected			Controls		
Body weight	Weight of ovary	Weight of oviducts	Body weight	Weight of ovary	Weight of oviducts
51.50	0.850	27.75	37.75	7.02	8.50
50.25	0.605	24.25	36.25	3.56	12.00
46.52*	0.425	14.02	33.50	5.55	7.75
44.75	0.415	14.72	28.70	3.57	8.01
42.62	0.495	17.95	28.45	3.74	7.15
41.00*	0.552	17.50	28.25	5.25	5.75
40.05	0.650	17.25	28.02	3.32	7.76
39.82*	0.575	13.25	24.55	4.52	7.25
39.61	0.625	13.27	23.15	3.25	5.48
39.25	0.425	14.02	23.10	3.09	6.32
38.87	0.385	15.05	20.55	2.57	5.50
34.35	0.425	9.61	20.15	3.74	3.85

* Oviposition occurred.

changes had taken place in the injected animals. All exhibited the typical changes found during the immediate pre-spawning period; that is to say the ova had been released from the ovary and had passed into the oviducts, whilst in the controls the ovaries remained intact and the oviducts empty.

The second series of frogs was injected with a volume of extract equivalent to 1 g. of fresh tissue, the animals being killed 7 days later. The results confirmed those of the first series. Out of 16 frogs, only 3 shed their eggs into the water, the total number not exceeding half a dozen. On *post mortem*, it was found that ovulation had occurred, however, in 12 animals.

Further experiments, though useless from the practical point of view, were carried out to see whether typical oviposition could be brought about by (a) further injections of anterior lobe substance, (b) subsequent injection of the oxytocic principle of the posterior lobe of the pituitary, (c) by placing injected animals in contact with males. All these were without result. It thus became clear that normal oviposition in *Rana* could not be taken to indicate activity of a preparation. It would be necessary to kill animals to confirm the result of an experiment. As the macroscopic appearance alone of the ovaries and oviducts in injected animals was sufficiently definite to indicate a positive result experiments were carried out to determine the earliest time that a reaction could be obtained. In Table II are shown the results of an experiment embracing 4 series of 8 frogs killed at different times after injection.

Table II.

Series	Time killed after injection hours	No. of frogs ovulating	Remarks
A	24	0	—
B	36	3	A few ova in body cavity. Ovary full
C	48	7	Body cavity full of ova. Ovary vestigial
D	72	7	Oviducts full. Several ova in body cavity. Ovary vestigial

The results show that a well-defined positive result can be obtained within 72 hours. If, however, this is taken to be liberation of ova from the ovary into the body cavity and not into the oviducts the reaction time can be reduced to 48 hours.

Several estimations of the gonad-stimulating power of anterior lobe preparations were carried out using this reaction.

Ovulation could be produced in 50 % of animals with a volume of suspension corresponding to as little as 0.25 g. of original tissue. It was also found that injection of acid extracts of the gland prepared by the writer's method [Bellerby, 1929] was effective.

The above experiments were carried out in the months of January and February. Subsequent investigation demonstrated a further disadvantage of *Rana*. The animal cannot be used in March or early April as it normally spawns during that time and ovulation can only be induced with a single injection from about the end of August to February. Experiments showed that during the rest of the year it was necessary to administer a series of injections to produce ovulation, the number of which varied according to the time of the year. As this phase of the work has been thoroughly investigated by Spaul [private communication] no further details are given here.

Apart from the indication of the drawbacks of *Rana* as an experimental animal two main points have emerged from the present enquiry. Firstly the results suggest that the process of ovulation and oviposition in *Rana* is more complicated than in *Xenopus*. It appears that some factor other than secretion of the anterior lobe of the pituitary is involved. In *Xenopus* the whole process of ovulation and oviposition can be attributed to anterior lobe activity. Secondly, *Rana* has one definite advantage over *Xenopus*. Because the ovary remains

undeveloped for several months it can be used to determine the effect of an extract in producing development of ova as distinct from one which induces their liberation from the fully developed ovary during that time. In *Xenopus* the ovary remains fully mature throughout the year when the toad is kept under optimum conditions. It cannot be used in consequence to demonstrate the former effect.

SUMMARY.

1. Ovulation without oviposition can be induced by injection of saline suspensions of anterior lobe pituitary tissue in the common frog *Rana temporaria*.
2. Oviposition only occurs in few injected animals and is never complete as in the normal animal.
3. Ovulation induced by single injections cannot be obtained during the whole year.

III. THE ACTION OF ANTERIOR LOBE PITUITARY EXTRACTS ON THE OVARY.

In a previous paper [Bellerby, 1933] and the foregoing part of the present paper it was shown that *Xenopus laevis* and *Rana temporaria* have certain advantages in work directed to extend knowledge of the chemical co-ordination between the ovary and the anterior lobe of the pituitary. In this paper are given details of the first of a series of observations made on the number and nature of the endocrine factors which are definitely concerned in the control of the ovarian cycle using *X. laevis* as the experimental animal. Before proceeding to the main issue discussed, it is necessary to refer to a matter which was not dealt with in the first paper of this series, namely the relation of external temperature to the ovulation process induced in *Xenopus* by extracts. Formerly, all experiments had been carried out at room temperature, but it transpired later that the temperature could be raised with advantage to the experimental procedure.

Relation of ovulation to external temperature.

Temperature variation might influence ovulation in two ways.

- (a) It might increase or decrease the time elapsing between injection and ovulation.
- (b) It might render the animal more or less sensitive to the effect of the extract so that different amounts would be required to produce the same effect.

Table I gives the results of a series of seven experiments regarding this point. Groups of 20 toads were divided into two batches of 10 each which were kept at different temperatures. They were injected with less than the quantity of acid extract required to produce ovulation in 100 % of the animals and received a threshold amount which under ordinary conditions would produce a positive response in about 60 % of animals. Toads were allowed to remain at their respective temperatures for four or five days before injection. As laboratory facilities did not permit of each batch being kept at a stipulated temperature, the difference for the two series was obtained by keeping one batch in a cold underground room and the other in a room heated with an electric fire. Both series were kept on a white background and under similar conditions of illumination.

It is clear that temperature has a marked influence in reducing the time taken for ovulation. In some hundreds of experiments which have been carried

Table I.

Temperature °C.	Temperature difference °C.	Number of toads ovulating				
		0-10 hrs.	10-24 hrs.	24-36 hrs.	36-48 hrs.	Total
31.5	15.5	7	1	0	0	8
16.0		0	4	3	0	7
27.0	11.0	6	0	0	0	6
16.0		0	0	5	3	8
24.0	10.0	4	0	0	0	4
14.0		0	1	5	0	6
26.5	12.3	7	0	0	0	7
14.2		0	3	3	0	6
25.2	10.7	3	4	0	0	7
14.5		0	4	4	0	8
26.2	7.7	4	2	0	0	6
18.5		0	7	0	0	7
23.5	7.0	5	2	0	0	7
16.5		0	2	2	0	4

out at room temperature (14-18°), ovulation under 12 hours only occurred once, the average time of occurrence being 18 hours.

In the animals kept at the high temperature (23-31°) ovulation took place in the majority of cases within 10 hours and in no case was it delayed beyond 48 hours. The average time was reduced to about 9 hours, all ova in some cases being shed within 7 hours of injection.

The results are summarised in Table II. It will be noted that a high temperature has no recognisable effect on the sensitivity of the response, practically the same number of toads ovulating at both temperatures. Temperature can therefore be disregarded in determining activity of extracts. At the same time the fact that a high temperature quickens the reaction increases the usefulness of *Xenopus* for purposes of assay.

Table II.

Temperature °C.	No. of toads injected	No. ovulating				Total
		0-10 hrs.	10-24 hrs.	24-36 hrs.	36-48 hrs.	
23.5-31.5	70	36	9	0	0	45
14.0-18.5	70	0	21	22	3	46

Relation of percentage response to amount of active substance injected.

A previous enquiry showed how it was possible to ensure a 100 % response with a sufficient dosage. In perfecting any method of assay based on the principle of the minimum dose, it is of the utmost importance to explore the range of dosage between quantities just sufficient to give a 100 % result and just too little to give any response at all. This point has been fully investigated in a series of experiments with acid extracts, the results being summarised in the graph shown in Fig. 1. Extracts were prepared as described in Part I of this series.

Roughly speaking a 50 % response requires about 4 times the absolute minimum dose and the least quantity required to evoke a 100 % response is 4 times the dosage required to produce any result at all. The reader may judge the reliability of this estimate by the very satisfactory consistency in the way the points arrange themselves in relation to a logarithmic curve. However, some attention was given to the reliability of each estimate in the mid region of the curve. In the neighbour-

hood of a 70 % response, an estimate based on 4 series of 10 toads is seen from the data in Table III to be subject to a maximum variation of 5 % when the whole is considered as a group of 40 animals. Each of the points in the graphs in Figs. 1 and 2 is based on 40 toads.

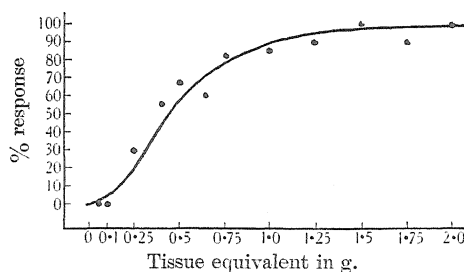


Fig. 1.

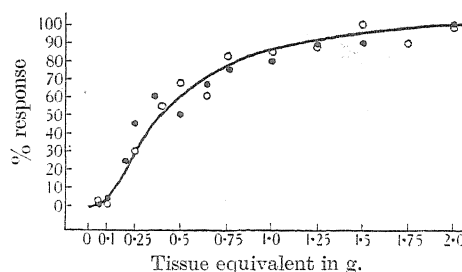


Fig. 2. ○ = Acid extract. ● = Alkaline extract.

Table III.

No. of batch	No. injected	No. ovulating	Percentage response		
			10 toads	20 toads	40 toads
A	10	3	30	65	70
B	10	10	100		
C	10	8	80		
D	10	7	70		

The action of alkaline extracts of anterior lobe.

The chief point which must be considered in any explanation of the mechanism by which the anterior lobe of the pituitary controls ovarian periodicity is whether one or more hormones are concerned. Evans and Long [1921] showed that injection of mature female rats with saline suspensions or extracts prepared with NaOH was followed by cessation of the oestrous cycle as a result of intensive luteinisation of the ovary. In the immature rat no effect was produced. Later Smith and Engle [1927] and Zondek and Ascheim [1927] found that injection of suspensions of anterior lobe tissue in the immature rat was followed by intensive follicular development, rupture of follicles and precocious oestrus. In the mature rat superovulation and superfoetation could be obtained [Engle, 1927]. At first the problem seemed a simple one. Basing his views mainly on the fact that injection of NaOH extracts in any dilution was ineffective in causing follicular growth and oestrus in the immature rat the writer suggested that two distinct substances were involved [Bellerby, 1928]. One was stable to alkali and on injection caused development of luteal cells; the other was stable to acid and induced growth and rupture of follicles. A similar view was expressed later by Evans and Simpson [1928] and Wiesner and Crew [1930]. However, further work [Bellerby, 1929] showed this view to be unsound. Making use of the rabbit, an animal in which no periodic changes take place in the ovary, it was found that injection of acid extracts of anterior lobe was followed by luteinisation as well as follicular growth and rupture. In fact, the injection of any anterior lobe preparation resulted in both luteinisation and follicular growth occurring collaterally to a corresponding extent.

Recently Fervold *et al.* [1931] and Claus [1931] claimed to have actually separated two substances from anterior lobe tissue with the specific physiological actions stated above. Subsequently, however, Van Dyke and Wallen-Lawrence

[1933] could not obtain evidence for the separation of the active substance into two fractions with solely follicle-stimulating or luteinising actions. Apart from this work no further reference is made to the numerous papers published on the "hormones of the anterior lobe of pituitary." The justification for this reticence is that in the majority of cases work on the existence of two or more substances in the anterior lobe transpires on further reading to be based on experiments in which no anterior lobe substance was employed. The large bulk of literature ostensibly dealing with the biochemical aspects of the anterior lobe is actually devoted to a detailed study of a certain novel but irrelevant attribute of the kidney. At the present time, therefore, no definite evidence is available to demonstrate the existence of two hormones directly influencing the ovary in the anterior lobe of the pituitary itself.

As stated in a previous paper, preliminary experiments showed that alkaline extracts were apparently just as effective as acid extracts in producing ovulation in *Xenopus*.

Extracts were prepared from ox pituitaries as follows.

The anterior lobes were dissected out within 4 hours of the death of the animal, weighed and ground to a fine paste with sand. The mixture was then extracted with $1\frac{1}{2}$ times its weight of $N/10$ NaOH for 24 hours at a temperature of 0° .

After first allowing the solid mass to thaw out it was carefully neutralised with 50 % acetic acid, phenol red being used as indicator. The mixture was then centrifuged and the red turbid extract decanted off. Injections were made without any delay into the dorsal lymph sac, strict attention being given to the points raised in the first paper.

In Fig. 2 is shown a curve plotted from experiments in which injection of alkaline extracts was carried out under the same conditions governing the results obtained with the acid extracts. Points obtained from the latter series are also included for purposes of comparison. Emphasis must be laid on the fact that ten out of twelve points on the alkaline curve were derived from experiments in which the tissue-equivalent dose given was the same as in the acid series. It will be seen that there is close similarity in the configuration of the two curves. No difference therefore is apparent in the effect of alkaline and acid extracts in producing ovulation in *Xenopus*.

CONCLUSIONS.

The work which has been done in the past seems to show that the ovary-pituitary relationship is common to all the land vertebrates, a fact which need evoke no surprise when we consider the widespread distribution of substances such as adrenaline (Cannon), secretin (Bayliss and Starling) and of the oxytocic and melanophore principles of the pituitary (Hogben and de Beer). In mammals the relationship is complicated by the highly complex specialisation of the follicle before and after ovulation. In amphibia this specialisation is lacking. For this reason the effect of pituitary extracts upon *Xenopus* is a valuable source of supplementary evidence when interpreting the data derived from the study of mammals alone. In the latter the dual action of anterior lobe extracts which evoke follicular growth and ovulation on the one hand and luteinisation on the other might be interpreted in two ways. One is that there are two substances respectively extracted more readily in acid or alkaline media. The other is that one and the same substance stimulates two processes which may be antagonistic. The assumption implied in the last remark would explain why on some occasions extracts have been found to evoke the first type of response and on other occasions the second.

A point of pivotal importance in the discussion is that both these processes, namely follicular development and ovulation on the one hand, and luteinisation on the other, affect different stages in the development of cells derived from the same primordial elements of the gonad. The action of extracts upon the ovary in *Xenopus* involves the response of cells derived from primordial elements with developmental potentialities of a simple order.

At this stage, although the data derived from the study of *Xenopus* do not permit us to make a confident decision in favour of either one or the other of the two hypotheses stated above, they do permit us to narrow the issues involved in the assumption of two separate substances. That in some experiments on mammals alkaline extracts have given luteinisation alone whilst other extracts have only evoked follicular development might be explained by those who invoke two separate substances in either of two ways. The first is that a luteinising substance which is more readily extracted in alkaline medium completely neutralises the action of another substance extracted both by alkaline and acid media, thus preventing the latter substance from evoking follicular development. The second is that such a luteinising substance does not directly neutralise the alternate component of an alkaline extract but stimulates the formation of cells with an antagonistic action so that the seat of the inhibition ultimately rests in the ovary itself.

In other words, the process of luteinisation may inhibit the response of immature follicles to the action of the follicular component. The fact that acid and alkaline extracts are equally effective in acting upon ovarian cells which cannot undergo a luteinising process definitely disposes of the first alternative. Thus we have now to decide in favour of the second alternative or of the hypothesis that only one substance is involved in the response of the ovary to anterior pituitary extracts. The latter is the more economical hypothesis and there are at present no conclusive objections to it. An underlying assumption in the previous remarks is that alkaline extracts which have been found by some workers to induce conspicuous luteinisation also contain the substance which promotes cell division of the primordial ovarian cells, culminating in the discharge of the ovum. If we prefer to accept the dual hypothesis this assumption is not only justified by the experiments on *Xenopus*. It is also implicit in the testimony derived from mammalian studies since the presence of numerous corpora lutea presumably involves the previous development of follicles.

In conclusion it will not be out of place to point out the relation of the present work on *X. laevis* and *R. temporaria* to similar studies on amphibia. The results obtained are entirely in accord with investigations on the induction of ovulation by means of pituitary transplants [Wolfe, 1929; Morgan and Sondheim, 1932; Noble and Richards, 1930; 1932]. They also confirm the work of Kehl [1930], Adams [1931] and Buyse and Burns [1931] who have induced ovulation by means of injection of extracts of mammalian pituitary substance. Thus no support is given to the contention that ovulation can only be induced in amphibia by homeo-transplants or extracts of glands from amphibia themselves [Houssay *et al.*, 1929]. The above investigations coupled with those carried out on mammalia clearly show that an ovary-stimulating substance in anterior lobe tissue has a wide distribution throughout the whole vertebrate series.

SUMMARY.

1. Alkaline extracts of anterior lobe pituitary injected in equivalent dosage throughout the whole range of effective concentration are as effective as acid extracts in producing ovulation in *Xenopus laevis*.

2. By keeping toads at a high temperature (23–31°) the time elapsing between injection and ovulation can be reduced from an average of 18 to 9 hours, thereby increasing the value of the animal for test purposes.

3. Temperature variation was not found to have any perceptible effect on the sensitivity of the response of ovulation to injection of extract.

The cost of animals and glands was borne by a Grant made to the Department by the Medical Research Council.

The work was carried out during the tenure of a personal grant made by the Birth Control Committee, to whom my best thanks are due.

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CCLXXVI. THE LIPOIDS OF SPLEEN AND LIVER IN VARIOUS TYPES OF LIPOIDOSIS¹.

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(Received November 4th, 1933.)

THE study of lipid distribution in derangements of lipid metabolism may be expected to throw new light on the physiology of lipid formation, transport and storage, just as the knowledge of carbohydrate metabolism is based to a great extent on the study of diabetes. At least three pathological entities of generalised "lipoid storage" disease or lipoidoses have been distinguished from clinical and pathological evidence and confirmed by chemical investigations: (1) Gaucher's splenomegaly, characterised by large deposits of the cerebroside kersin in spleen and liver; (2) Niemann-Pick's disease, in which the phosphatide and cholesterol contents of the viscera, the bone-marrow and the brain are increased at the expense of neutral fat; (3) Schüler-Christian's disease with replacement of bone tissue by cholesterol deposits. The outstanding chemical characteristics of these diseases have been described in case reports in the medical literature. However, since the methods used and the condition in which the organs are obtained from the *post mortem* room leave much to be desired from the point of uniformity, we took advantage of the simultaneous availability of fresh organs from several cases of these relatively rare conditions and studied the lipid distribution in spleen and liver of three cases of Gaucher's disease, two cases of Niemann-Pick's disease, and three control cases. As the methods of extraction have been discussed in another connection [Sobotka *et al.*, 1930; 1932] they will be only briefly described in the experimental part.

The synopsis in Table I shows that three cases of Gaucher's disease give concordant results, namely, shifts from the phosphatide fraction to the neutral fat fraction, and possibly an increase of cholesterol in comparison with the control analyses. The main characteristic consists in the increase of the balance in the acetone-insoluble lipid mixture after allowance is made for cholesterol and phosphatides. This increase is due to the presence of considerable amounts of the pathological cerebroside kersin [Lieb, 1924]. The cerebroside was isolated following Rosenheim's procedure [1914; 1916]. Spheroliths of kersin, completely free from phrenosin, were identified by Rosenheim's test with the selenite plate under the polarising microscope and 5 g. pure kersin were prepared from 52 g. of pooled acetone-insoluble material of spleen and liver (cases C and T).

The analysis of case H of Niemann-Pick's disease revealed remarkable increases of phosphatides and cholesterol esters over normal and the almost complete absence of neutral fat. The latter finding was duplicated in another case of Niemann-Pick's disease the analysis of which we owe to Dr J. Kahn, who will communicate his findings in detail elsewhere [1933]. A new feature in this

¹ Read before the Section of Medicinal Chemistry at the Semiannual Meeting of the Amer. Chem. Soc., Chicago, September 1933.

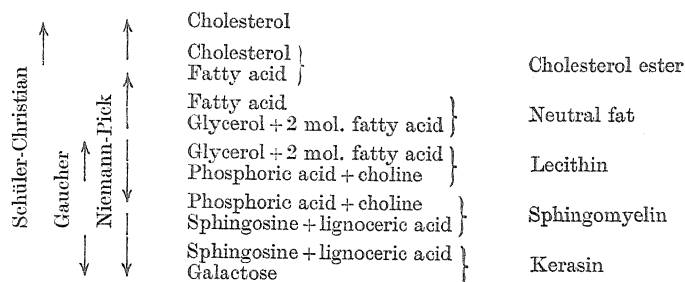
Table I. *Synopsis of lipid analyses.*

		Percentages of total lipoids									
	Case	Weight moist g.	Lipoids g.	Tot. lip. Tot. sol.	Free chol.	Chol.- ester	Phospha- tides	Neutral fat + E.A.	Balance (Cerebr.)	Free chol. Tot. chol.	Fatty acids
Spleens											
	Gaucher T	2615	280	50.6	11.6	0.5	17.7	33.6	36.6	97	44.8
	Gaucher C	2100	132	31.7	6.5	7.5	33.5	26.4	26.1	60	51.6
	Gaucher B	305	19.4	30.5	4.0	10.6	21.9	35.6	27.9	39	54.2
	Gaucher average	—	—	—	7.4	6.2	24.3	31.9	30.2	—	50.9
	Niemann-Pick H	180	14.7	45.8	9.6	6.9	62.6	1.2	19.7	70	47.9
	Niemann-Pick (Kahn)	205	25.9	58.5	25.8	11.7	21.8	2.3	38.4	80	22.5
	Controls (3)	—	—	13.6	10.1	1.5	41.5	25.9	21.0	91	54.7
Livers											
	Gaucher T	3400	252	32.1	7.5	11.5	26.6	22.7	31.7	67	45.5
	Gaucher C	4100	304	34.3	9.6	11.5	15.5	33.1	30.3	58	47.6
	Gaucher B	405	45.6	46.6	9.7	6.4	31.1	27.1	25.7	72	50.7
	Gaucher average	—	—	—	8.9	9.9	24.4	27.6	29.2	—	47.9
	Niemann-Pick H	500	60.5	33.4	4.9	11.3	67.9	0.3	15.6	47	46.1
	Niemann-Pick (Kahn)	180	20.6	41.7	20.4	6.9	28.5	7.8	36.4	84	30.5
	Controls (2)	—	—	23.3	4.9	2.3	39.1	24.3	29.4	79	51.7
Brains											
	Gaucher B	805	50.1	38.4	8.6	42.6	23.8	17.3	7.7	25	51.8
	Niemann-Pick H	630	48.8	45.6	3.4	25.5	53.5	0.0	17.6	18	48.7

case was the great increase in cholesterol. One portion of the acetone extract consisted practically of pure cholesterol which crystallised in large characteristic colourless platelets from the pigmented fluid. Cholesterol ester was almost absent. Parallel with this increase and with the decrease in the amount of neutral fat, is the reduction in the quantity of phosphatides, especially pronounced in the spleen. The increase in the balance of the acetone-insoluble fraction (*minus* cholesterol and phosphatides) is at least partly due to the presence of kersin of which 1.8 g. could be crystallised and identified; the presence of phrenosin was definitely excluded. Products of partial hydrolysis of phosphatides as phosphatic acids [Chibnall and Channon, 1927] and of cerebrosides such as psychosin [Thudichum, 1884] and acyl-sphingosines might contribute to this fraction.

The common fatty acids, stearic, palmitic and oleic acids, are presumably distributed between cholesterol ester, neutral fat and monoaminophosphatides (lecithins and kephalins), while the higher acids, especially lignoceric acid, are found in diaminophosphatides (sphingomyelin) and in cerebrosides. Thus, we may estimate the amount of the ordinary fatty acids from the percentages contained in cholesterol ester (43.3 % oleic acid), phosphatides (70.3 % for stearo-oleocithin) and triglycerides (95.6 % for stearopalmito-oleoglyceride). Any free fatty acid, originally present or formed by decomposition, would be included in the figure for neutral fat according to the procedure of extraction. As will be seen from the last column of Table I, the total amount of fatty acid varies around 50 % for all cases investigated with the exception of Kahn's case.

The lipoids of animal tissues are intimately interlocked with each other through the presence of common groups as exemplified in the following scheme. The interconversion of lipoids can thus be interpreted as "exchange esterification" brought about by the hydrolytic and synthetic activity of esterases [Sobotka, 1930]. In the lipid disorders under investigation, the enzymic regulation of these equilibria seems to be disturbed. Substances, useful under normal conditions as transport forms, are not further metabolised, but are apt to clog the organs, especially those of the reticuloendothelial system. This is the case in Gaucher's disease with kersin, while in Niemann-Pick's disease (case H), lecithin and cholesterol ester accumulate at the expense of neutral fat as indi-



cated in the scheme above by the arrows. This process went even further in Kahn's case with subsequent depletion of the common fatty acids.

Liver esterase. A study of the esterase in the liver specimens strongly supports the assumption of enzymic deficiency. The esterase contents of twelve livers were determined by the saponification of methyl butyrate. Four livers from cases of Gaucher's disease and one liver from a case of Schüler-Christian's disease contained diminished amounts of esterase when compared with three normal livers (accidental violent death). Liver esterase was also diminished in a case of fatty degeneration (alcoholism) and in an atrophied specimen, and it was almost absent in tuberculosis. The liver of Kahn's case of Niemann-Pick seemed to contain no esterase at all while the liver of our case H was not included, as this enzymic study was begun in the later course of the work.

EXPERIMENTAL.

The fresh organs were chopped and extracted with cold acetone and subsequently in a Soxhlet apparatus with hot acetone, ether and finally with alcohol. White matter precipitated from the hot acetone extract upon cooling. Ether and alcohol extracts from the same organ were combined for analysis. All fractions were analysed for total and free cholesterol, phosphorus and Kjeldahl nitrogen. The results for the cold acetone extract and for the supernatant of the hot acetone extract were combined. Allowance was made for the fatty acid bound with cholesterol, and the balance—after deduction of the weights of cholesterol, cholesterol esters and phosphatides calculated as lecithin—was assumed to consist of neutral fat and free fatty acid. The analytical results for the white matter from the hot acetone extract were added to those for the ether *plus* alcohol extract. The amounts not accounted for as cholesterol or phosphatides comprise the cerebrosides and other less soluble phosphorus-free lipoids. From these fractions kerasin was isolated, if present, according to Rosenheim's procedure. The white matter was extracted with tetralin [Page, 1930]; the tetralin extract was poured into excess acetone, and the precipitate was extracted with light petroleum, leaving all phosphatides behind. The material soluble in light petroleum was recrystallised from a mixture of equal volumes of chloroform and alcohol and from 90 % acetone. The white substance obtained had $[\alpha]_D = -9.2^\circ$; it contained 1.96 % N (calculated for kerasin, 1.73) and yielded on hydrolysis 23.0 % (calculated, 22.2) sugar by Bertrand's method, calculated for galactose. It contained less than 0.05 % phosphorus and crystallised from pyridine in spheruliths which proved to be pure kerasin in the selenite plate test.

The measure of esterase is the esterase unit (E.U.), equal to that amount of enzyme which hydrolyses 0.2 g. of methyl butyrate in 20 cc. of a buffer solution to an extent of 25 % in 60 minutes at 30° . 8 cc. of a $M/3$ buffer mixture of $(\text{NH}_4)_2\text{HPO}_4$ and NH_3 in the proportion 9.5 to 0.5 were used for 20 cc. reaction

mixture making the initial p_H 8.2. The amount of hydrolysis was determined by titration with $N/5$ NaOH with thymolphthalein as indicator.

One part chopped fresh liver was allowed to stand for 7–14 days with 3 parts glycerol. The strained glycerol extract was used in a dilution equivalent to 100 mg. fresh liver or 25 mg. dry liver tissue for 20 cc. of reaction mixture. The enzymic strength of a material was defined by Willstätter and Memmen [1924] by its esterase value (E.V.) which equals the amount of E.U. in 10 mg. Thus, the amount of E.U. calculated in the above tests from the amount of butyric acid liberated, multiplied by 10/25, gives the E.V. of a specimen. Aqueous ammoniacal extracts of acetone-dried liver tissue are always weaker than the glycerol extracts from fresh liver.

The E.V. of three normal livers were 1.76 (0.54 for acetone-dried sample), 1.60 and 1.42, those of the Gaucher cases 0.69 (T), 0.27 (C), 0.23 (B), and 0.80 (Bayonne; acetone-dried sample, 0.16). In the last case, Gaucher's disease was an incidental *post mortem* finding in an accidental death. The liver from a child with Schüller-Christian's disease had an E.V. of 0.27, that from a case of Niemann-Pick's disease (Brooklyn) of which only an acetone-dried specimen was available, had no activity whatsoever. Other diseased livers, examined for comparison, included fatty degeneration with E.V. 0.57, a liver atrophy with E.V. 0.55, and a tuberculous liver with the low E.V. of 0.13.

SUMMARY.

The lipoids of liver and spleen in three cases of Gaucher's disease were analysed and compared with the findings in two cases of Niemann-Pick's disease and with normals. The classification of these diseases according to the chemical nature of the lipoids deposited is criticised. The various shifts in lipid distribution are explained by deficiency of lipolytic enzymes. Deficiency of liver esterase is demonstrated experimentally in these and similar cases.

Our thanks are due to Dr W. Antopol, Bayonne Hospital, Bayonne, New Jersey, for a liver of Gaucher's disease, Drs M. A. Goldzieher and J. Kahn, Beth Moses Hospital, Brooklyn, N.Y., for the liver of Niemann-Pick's disease and for the communication of their analytical results, to the Chief Medical Examiner of the City of New York, Dr Charles V. Norris, for three normal livers, and to Prof. W. M. Sperry of the Presbyterian Hospital, N.Y.C., for the liver of Schüller-Christian's disease.

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CCLXXVII. CUTANEOUS BLOOD-SUGAR CURVES AFTER THE ADMINISTRATION OF FRUCTOSE, MANNOSE AND XYLOSE.

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(Received November 2nd, 1933.)

THE oral administration of galactose to man results not only in the finding of galactose in the blood-stream but of an increased concentration of glucose (estimated as fermentable sugar) [Harding and Van Nostrand, 1929-30; Roe and Schwartzmann, 1932; Harding and Grant, 1932-33]. The increases in blood-glucose are variable in amount but can usually be found in arterial blood, if a sufficient number of samples be taken. In the fasting rabbit and the rat neither Corley [1927] nor Cori and Cori [1928] had found such increases, though Blanco [1928] reported them in the well-fed rabbit. The conditions and methods of these last three observations make them non-comparable with the findings on man. A previous meal of carbohydrate or fat, however, appears to cause no difference in the human results.

The oral administration of fructose in man is also presumably accompanied by increases in blood-glucose. Isaac [1920] first emphasised this possibility, pointing out that the increases in total blood-sugar during fructose tolerance tests by no means represent the amount of circulating ketose. Direct determination of blood-fructose by modifications of the Selivanoff reaction [Folin and Berglund, 1922] or by the Van Creveld reaction [Kronenberger and Radt, 1927; Radt, 1928] show that the amount of circulating fructose, after fructose ingestion, in normal man is very small, and that such amounts do not account for the observed increases in total blood-sugar. In the rabbit Corley [1929] observed the same discrepancy.

Are the increases in blood-glucose following the administration of galactose and fructose to man evidence of a general stimulation or irritation of a glucose-producing mechanism, due to the presence in the blood-stream and tissues of a large amount of a foreign sugar? Or do they represent the conversion of the entering sugar into glucose, either directly or through some intermediate? Harding and Grant [1932-33] concluded that blood-sugar studies after galactose administration gave evidence too limited and uncertain in character. To some extent the former view may be true, for Fishberg [1930] found the intravenous injection of galactose or xylose into the rabbit to produce a sharp increase in blood-fermentable sugar, though Corley's results with *l*-arabinose and *d*-xylose are less clear [1926; 1928]. Orally administered, neither pentose produces an increase in blood-glucose in the rabbit [Corley, 1926; 1928].

As both the amount of foreign sugar entering the blood-stream and its nature are likely to influence the result we have examined the blood-sugars in man after

the oral administration of varying doses of fructose, mannose and xylose. The use of *Proteus vulgaris* [Harding and Nicholson, 1933] forms an exact method for the separation of glucose from fructose or mannose. Glucose and xylose can be separated by the same method or by the action of baker's yeast. The methods allow of the use of a small amount of cutaneous blood and thus of the collection of a reasonable number of blood specimens. Whilst fructose tolerance tests in man have been frequently reported there are no records of the use of mannose and no blood-sugar observations after the oral administration of xylose to man. We have compared the results with those observed after 40 g. and 80 g. galactose by Harding and Grant [1932-33].

ANALYTICAL METHODS.

Separation of glucose from fructose or mannose in cutaneous blood.

0.5 cc. finger blood is laked in 8.5 cc. water in a centrifuge-tube, 0.5 cc. 10 % sodium tungstate and 0.5 cc. 0.66 N H_2SO_4 are successively added from accurate micro-burettes, the mixture well stirred, allowed to stand 10 mins. and centrifuged. The centrifugate is decanted and by means of a knife blade sufficient solid Na_2HPO_4 is added to adjust the p_H to 6.7-7.2. 3 cc. centrifugate are added to 0.25 g. wet-weight *Proteus vulgaris* [Harding and Nicholson, 1933] contained in a flat, high-efficiency tube belonging to the Lündgren high angle centrifuge and incubated with agitation for 30 mins. at 38° . The bacillus is separated in the centrifuge and the supernatant fluid decanted. The residual reducing substances are determined in 1 cc. of this fluid by mixing with 1 cc. of the sensitive copper reagent [Harding and Downs, 1933], heating for 10 mins. in a rapidly boiling water-bath, adding 1 cc. 1 % KI solution and 1 cc. $N H_2SO_4$, allowing to stand with occasional agitation for 3 mins. and titrating the excess iodine with 0.0025 N thiosulphate. At the same time, the total sugar is determined in 1 cc. of the original Folin-Wu centrifugate. The difference represents glucose. The value for the residual reducing substances in the fasting specimen of blood is subtracted from the values obtained after fructose or mannose administration. This gives the fructose or mannose value in cc. 0.0025 N thio-sulphate.

The method follows that used by Harding and Grant [1932-33] for the fractionation of glucose, galactose and residual reducing substances. It was shown that the residual reducing substances after removal of glucose and galactose in a Folin-Wu blood-filtrate remained very constant during galactose tolerance tests. The constancy of this non-sugar fraction has been assumed in the present work. As control, we have occasionally determined the total glucose-fructose fraction by using *Monilia krusei* to remove both sugars. The total as determined in this manner agrees satisfactorily with that obtained by the method just detailed. Added fructose is satisfactorily determined. It is absolutely essential to control the p_H of the Folin-Wu filtrate by the addition of Na_2HPO_4 . The p_H of a Folin-Wu filtrate prepared with the usual larger quantities of venous blood is very constant and about 6.8. In these filtrates we never experienced any difficulty in separating glucose from fructose or mannose. The p_H of corresponding centrifugates from the small amounts of capillary blood is quite variable, sometimes being less than 6.0. The *Proteus* does not completely remove glucose under these conditions.

Separation of glucose and xylose in cutaneous blood.

The procedure detailed above can be used if desired, but owing to the difficulties connected with the use and lack of keeping qualities of *Proteus*, it is more convenient to use 0.1 g. wet-weight washed baker's yeast. This removes glucose quantitatively from the xylose.

The determination can also be made by allowing the yeast action on whole blood to precede the precipitation of proteins by the Herbert and Bourne technique. In one centrifuge-tube is placed 0.25 g. washed baker's yeast. To this and to a second centrifuge-tube are added 2.5 cc. of a solution containing 3 % sodium sulphate and 0.6 % sodium tungstate. 0.3 cc. of blood is placed in each tube and the contents well mixed. The yeast-containing tube is now incubated for 8 mins. at 38° and then to each is added 0.2 cc. 0.5 N H_2SO_4 . The contents of both tubes are very thoroughly stirred, centrifuged and decanted, and the reducing value is determined on 1 cc. samples from each tube. The value of the yeast-containing tube represents xylose when a small correction (about 3 mg./100 cc.) is applied for non-sugar reducing material. The difference between the values of the two tubes is glucose.

Examination of urine for fructose or mannose.

The urine is diluted to an appropriate degree, usually 5 or 10 times, if negative to Benedict's reagent. The diluted urine is treated with H_2SO_4 and Lloyd's reagent [Harding and Selby, 1931]. The p_H of the filtrate is corrected to 7.0 by the addition of solid Na_3PO_4 . 15 cc. of the above neutral filtrate are poured onto 0.75 g. wet-weight washed *P. vulgaris* and incubated for 30 mins. at 38° with stirring, 0.3 g. NaH_2PO_4 is added and the mixture centrifuged in the high angle centrifuge. 0.6 g. light calcined MgO is added to the centrifugate which is then shaken for 15 mins. The mixture is filtered and the filtrate adjusted to p_H 7.0 by a few drops of 1 : 2 H_2SO_4 from a very thin glass rod. The reducing power is determined on 1 cc. (as in the previous blood-filtrates) and represents the fructose or mannose *plus* the usual non-fermentable "sugar". 3 cc. of the filtrate from the above MgO treatment are poured onto 0.5 g. wet-weight *M. krusei* [Harding and Nicholson, 1933] in a centrifuge-tube, incubated for 30 mins. at 38° with stirring, centrifuged and the reducing power determined on 1 cc. of the centrifugate. The difference between this and the previous determination represents fructose or mannose. If only small amounts of known fructose or mannose are present baker's yeast can be substituted for *M. krusei*.

Determination of xylose in urine.

No specific method is available for xylose determination. Corley [1926] has used extra non-fermentable sugar after xylose administration as an index of excretion. McCance and Madders [1930] have estimated xylose in urine as extra pentose, or furfuraldehyde-yielding material. After xylose administration to man, the amount appearing in the urine is usually sufficient to give a positive Benedict's test. The urine thus requires a high dilution, and it is sufficiently accurate to term xylose any non-fermentable "sugar" left after treatment with yeast.

The factors requisite for the conversion of cc. 0.005 N thiosulphate to fructose, mannose or xylose can be found in Table I of Harding and Downs [1933].

EXPERIMENTAL.

Normal male subjects fasted from 6.0 p.m. to the next morning. The bladder was emptied at 9.0 a.m. The desired amount of sugar dissolved in

400 cc. water was taken. Finger blood-samples were drawn every 15 mins. during the first hour, and at 30 min. intervals during the second hour. Urine was collected at 11.0 a.m. The sugars were bought as pure, but the xylose was found to contain a small amount of reducing substance removable by yeast.

RESULTS AND DISCUSSION.

Fructose. The amounts of fructose appearing in the arterial blood after ingestion of 50 g. are very small. The small amounts, determined by the use of micro-organisms as a method of analysis, agree in general with those obtained by colorimetric chemical methods (Fig. 1). The main increases which occur in

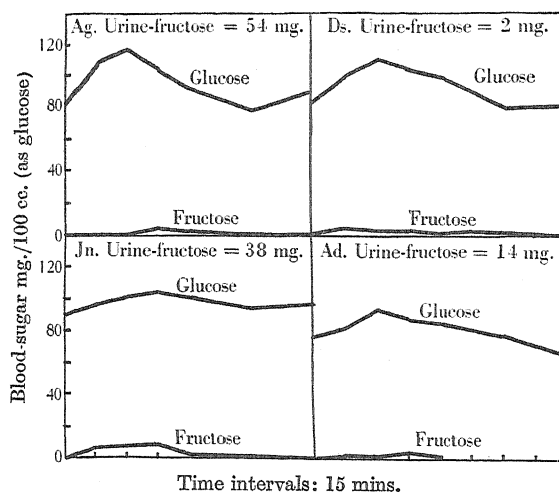


Fig. 1. Showing glucose and fructose in cutaneous blood after oral administration of 50 g. fructose.

the total blood-sugar are due to glucose. Similar, though perhaps not so large, increases are found after the ingestion of 25 g. fructose (not shown). Small amounts of fructose were also found in the urine, varying from 2 to 54 mg. in the two-hour period. These results support the supposition of Harding and Selby [1931] that part of the fermentable sugar of a daily urine can be fructose, arising from its ingestion in the form of invert sugar in fruits, honey, etc.

Mannose. The blood analyses after ingestion of either 25 or 50 g. mannose are devoid of positive findings (Fig. 2). Neither the glucose nor the mannose fraction showed variations outside the biological or experimental variation. Of the so-called fermentable sugars, mannose shows the lowest rate of absorption. Using Cori's [1925] figures on the rat as a general guide, mannose is absorbed from the intestine at slightly less than one-half the rate of fructose, yet this cannot be advanced as the entire reason for our failure to demonstrate its presence in the blood-stream. Some absorption unquestionably took place, for its presence in small amounts was readily shown in the urine. Moreover, the presence of xylose, which in the same series possesses only a very slightly inferior absorption rate, can readily be shown in arterial blood after its oral ingestion. The rate of utilisation of mannose must equal or exceed its rate of absorption. The few mg. which escape into the urine provide evidence for the

practical non-existence of a renal threshold for mannose. From our results on fructose the renal threshold for this sugar must also be non-existent or extremely low [Folin and Berglund, 1922]. The renal threshold for galactose appears to be equally low [Folin and Berglund, 1922; Harding and Van Nostrand, 1929-30; Harding and Grant, 1932-33], and glucose thus seems to be the only utilisable sugar which shows the interesting phenomenon of a high renal threshold.

Xylose. This sugar is not utilised by the mammalian organism. True, coefficients of utilisation have been calculated from time to time on this and other pentoses, but the use of the term utilisation is based upon a lack of complete recovery of an administered amount, rather than on definite proof that the sugar

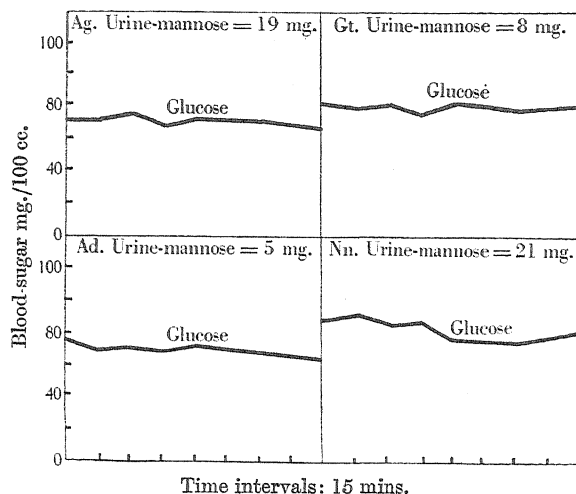


Fig. 2. Showing the lack of rise of blood-glucose after oral administration of mannose. Mannose was not detected in the blood. Subjects Ag and Gt received 25 g. mannose; subjects Ad and Nn 50 g.

takes an active part in daily metabolism. In recent studies Miller and Lewis [1932] have been unable to demonstrate satisfactory glycogen formation in the rat. Very active tissue, such as tumour tissue [Russell, 1923] or embryonic kidney tissue [Watchorn and Holmes, 1931], causes the disappearance of xylose from a nutrient solution, coincidentally with an increase in area of the growth, but the mechanism of the utilisation of xylose evidently differs from that accompanying the similar disappearance of glucose, fructose and galactose. The loss of xylose from the nutrient solution is not accompanied by any diminution of production of urea and ammonia as is the case invariably with glucose or fructose, and irregularly with galactose [Watchorn and Holmes, 1931].

The curve of blood-xylose after the oral administration of 50 or 25 g. differs from that of the other sugars. Large amounts are present, and at the end of two hours there is only a slight diminution in its concentration in three of the four experiments. Even after ingestion of only 10 g. the curve of blood-xylose has not reached zero level in two hours (Fig. 3). The contrast between the blood-sugar curves of this sugar and galactose (where the zero level is reached in two hours after ingestion of 40 g.) emphasises their different metabolic behaviour. Galactose belongs to the class of utilisable sugars, a fact which is now being more widely recognised than formerly.

Despite the large amounts of xylose in the blood-stream there is no marked rise in blood-glucose. The amounts of non-glucose sugar are larger than after either galactose or fructose. Only after ingestion of 50 g. of xylose is there, in one subject, any increase in blood-glucose which might be legitimately reckoned as outside the normal fasting variation. The presence of a considerable quantity of foreign sugar in the blood-stream evidently need not produce a hyperglycaemia. These results on man agree with those of Corley on the rabbit. The amounts of xylose found in the urine are comparable with those noted by McCance and Madders [1930].

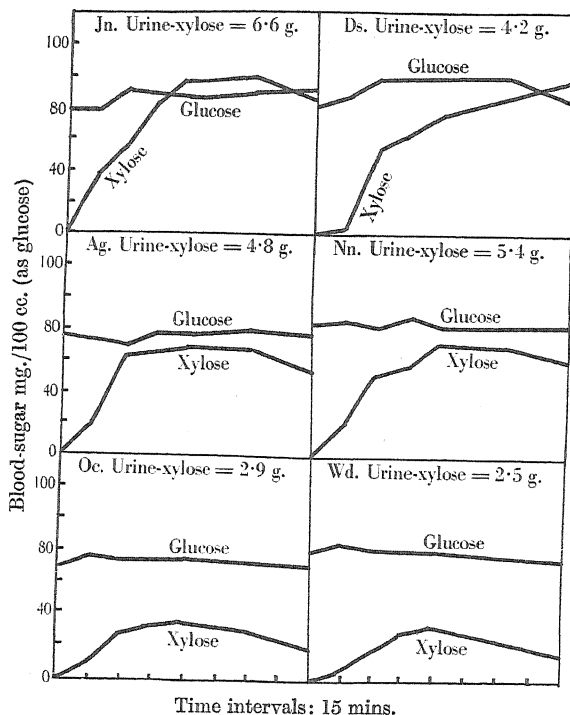


Fig. 3. Showing glucose and xylose in cutaneous blood after oral ingestion of xylose. Subjects Jn and Ds received 50 g. xylose; subjects Ag and Nn 25 g.; subjects Oc and Wd 10 g.

Galactose. Figs. 1 and 2 in the previous paper of Harding and Grant [1932-33] show the average curves of blood-galactose and glucose after the ingestion of 40 and 80 g. galactose by man.

What significance must be attached to the increase in blood-glucose occurring after the oral administration of galactose and fructose? Harding and Van Nostrand, and Roe and Schwartzmann, attributed it to direct conversion of galactose into glucose. Harding and Grant felt, however, that the increases were too variable and too independent of the amount of galactose to have their origin in a direct simple interconversion of the sugars. Yet, as we have just seen, the presence of similar amounts of foreign sugar in the shape of xylose will not produce a corresponding hyperglycaemia. Galactose and fructose are apparently a class apart, and produce a hyperglycaemia as a result of some specific action.

The most rational explanation to adopt at present seems to be to regard the hyperglycaemia as a result of the production of some precursor of glucose. Fructose has been known to produce lactic acid on ingestion in man [Campbell and Maltby, 1928] and galactose has recently been shown to give rise to lactic acid in dogs [Wierzuchowsky and Laniewski, 1931]. Whether the production of lactic acid from these two sugars reflects the primary path of metabolism or a secondary one is immaterial. Any large production would give rise to a temporary hyperglycaemia. Mannose is perhaps too slowly absorbed to give rise to a large amount of any glucose-former. Xylose, as a non-utilisable sugar, would possess no such action.

While such a view may harmonise the facts as known after the oral ingestion of non-glucose sugars, it would seem necessary to assume that extremely large amounts, such as are temporarily introduced into the blood-stream by intravenous injection, can give rise to hyperglycaemia, independently of metabolic change, as suggested by Fishberg. The one subject of our xylose series who showed a very slight hyperglycaemia after ingestion of 50 g. may have been at the commencement of such a change. The high dosage, however, produced a strong diarrhoea and precluded further experiments.

SUMMARY.

1. The oral administration of fructose to man produces an increase in blood-glucose. This is similar to the behaviour of galactose. Small concentrations of blood-fructose were also measured.

2. The micro-organism method of analysis shows small amounts of urinary fructose after fructose feeding.

3. It was not possible to demonstrate either the presence of mannose in the blood-stream or a hyperglycaemia after the oral administration of mannose. The slow rate of absorption of this sugar, coupled with a rapid utilisation, may account for these results.

4. Mannose was detected in the urine after ingestion of mannose.

5. The oral administration of xylose results in large amounts of circulating xylose in the blood. The rate of removal of xylose from the blood must be small, as easily measurable amounts are found at the end of two hours even after the ingestion of only 10 g.

6. Large amounts of xylose are found in the urine after ingestion of xylose.

7. It is suggested that the hyperglycaemias following the ingestion of fructose and galactose are the results of the production of a glucose-precursor. Any foreign sugar, however, if its blood concentration is sufficiently great, may produce a hyperglycaemia.

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CCLXXVIII. IRRADIATED ADENINE SULPHATE AS A SOURCE OF VITAMIN B₁ FOR GROWTH¹.

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(Received September 25th, 1933.)

EVER since the early findings of Suzuki *et al.* [1912] and of Drummond and Funk [1914] that nicotinic acid is a constituent of the curative fraction of rice polishings, suggestions have come forth associating the antineuritic factor with substances having purine or pyridine structures. The latter idea was accepted as a working basis by R. R. Williams [1916]. Williams, however, believed that only certain isomerides of the hydroxypyridines were effectual in curing polyneuritis in pigeons and, hence, developed his theory of the rôle of isomerism in the physiology of vitamins. This work was not corroborated by other investigators.

In a recent preliminary communication Tschesche [1933] has called attention to the similarity between adenine hydrochloride and the vitamin B₄ crystals isolated by Barnes *et al.* [1932]. Heard *et al.* [1933], while admitting that vitamin B₄ crystals consist largely of adenine, stated that the latter did not exhibit vitamin B₄ activity. Neither did irradiated adenine produce a vitamin B₄ response. Contrary to the experience of the latter workers, Guha and Chakrovarty [1932] have reported in a preliminary communication sent by cable from India, that vitamin B₁ can be synthesised photochemically by the ultra-violet irradiation of adenine sulphate.

In our recent investigations of the protective action of vitamin B₁ against the toxicity of thyroxine, highly concentrated extracts prepared by the author were employed, which were potent to the extent that 1 mg. daily per animal produced a growth of 3 g. weekly, which is equivalent to the modified Sherman unit [Sherman and Spohn, 1923]; and 3 to 5 mg. per rat daily produced a growth of 12 to 15 g. weekly, which is equivalent to the Chick and Roscoe unit [1929]. Since in our recent hyperthyroidism work [Sure and Smith, 1933] we have employed pure crystalline thyroxine, it was desirable to follow such studies with as concentrated and as pure a source of vitamin B₁ as is obtainable. Attempts were made, therefore, to secure vitamin B₁ by the procedure of Guha and Chakrovarty. Since no details of the technique were given, adenine sulphate² was irradiated for periods varying from 1 to 30 mins. These time limits had been found adequate for various sources of vitamin D. The irradiated adenine sulphate was dissolved in water and the daily dose administered volumetrically with a finely graduated pipette.

In view of the recent report of Kinnersley *et al.* [1933] that as little as 0.1 γ (0.0001 mg.) of pure vitamin B₁ should be a sufficient daily dose for pigeons, a smaller daily allowance than 0.1 mg. for the albino rat was not tried. The daily

¹ Research paper No. 318, Journal Series, University of Arkansas.

² A pure product purchased from Eastman Kodak Company, Rochester, N.Y.

Table I. *Irradiated adenine sulphate as a source of vitamin B₁ for growth.*

Change in body weight is expressed in g. (D)=died.

Animal number and sex	Period of experimen- tation (days)	Daily dosage (mg.)	Period of irradiation				
			1 min.	5 mins.	10 mins.	16 mins.	30 mins.
♀ 9649	13	0.1	—	—	-9(D)	—	—
♀ 9675	13	0.1	—	—	-8	—	—
	7	0.5	—	—	-4	—	—
♂ 9676	13	0.1	—	—	-5	—	—
	7	0.5	—	—	-2	—	—
	7	0.1	-7	—	—	—	—
	14	0.5	-8(D)	—	—	—	—
♀ 9677	13	0.1	—	—	-11	—	—
	7	1.0	—	—	-11	—	—
♀ 9678	13	0.1	—	—	-6	—	—
	7	1.0	—	—	-8(D)	—	—
♂ 9694	13	0.1	—	-4	—	—	—
	7	0.1	—	—	—	—	-15(D)
♀ 9695	13	0.1	—	-6	—	—	—
	7	0.1	—	—	—	—	-4
	7	0.1	-2	—	—	—	—
	7	0.5	-4(D)	—	—	—	—
♂ 9696	13	0.1	—	-8	—	—	—
	7	0.2	—	—	—	—	-8
♀ 9697	7	0.2	—	—	-4	—	—
	7	0.2	—	—	—	—	-3
	7	0.1	-1	—	—	—	—
	28	1.0	-9(D)	—	—	—	—
♀ 9698	7	0.2	—	—	-1	—	—
	40	0.5	—	—	—	—	-17(D)
♀ 9707	7	0.2	—	—	+1	—	—
	7	0.2	—	—	—	—	-2
	8	0.2	-4(D)	—	—	—	—
♂ 9711	7	0.2	—	—	—	—	-5(D)
♀ 9718	7	0.2	—	—	-3	—	—
	7	0.5	—	—	-9	—	—
♀ 9719	7	0.2	—	—	-1	—	—
	7	0.5	—	—	-9	—	—
♂ 9720	7	0.2	—	-2	—	—	—
	7	0.5	—	-4(D)	—	—	—
♂ 9721	7	0.2	—	-7(D)	—	—	—
♂ 9725	13	0.1	—	-5	—	—	—
	7	0.5	—	-10(D)	—	—	—
♀ 9726	6	0.1	—	-6	—	—	—
	7	0.1	—	—	—	-2	—
	15	0.5	—	—	—	-6	—
	7	0.5	—	—	—	—	-7(D)
♀ 9727	6	0.1	—	±0	—	—	—
	7	0.1	—	—	—	-6	—
	7	0.5	—	—	—	-10(D)	—
♂ 9728	6	0.1	—	-7	—	—	—
	7	0.1	—	—	—	-3(D)	—
♀ 9729	6	0.1	—	±0	—	—	—
	7	0.1	—	—	—	-2	—
	15	0.5	—	—	—	-5	—
	18	1.0	—	—	—	-5(D)	—

Table I (cont.).

Animal number and sex	Period of experimentation (days)	Daily dosage (mg.)	Period of irradiation				
			1 min.	5 mins.	10 mins.	16 mins.	30 mins.
♀ 9730	16	0.1	—	—	—	+2	—
	11	0.1	-6	—	—	—	—
	19	1.0	-8(D)	—	—	—	—
♂ 9731	13	0.1	—	—	—	±0	—
	3	0.1	—	—	—	—	-3
	9	0.1	-9(D)	—	—	—	—
♀ 9733	13	0.1	—	—	-5	—	—
	7	0.1	—	—	—	—	-3(D)
♀ 9734	13	0.1	-4(D)	—	—	—	—
♂ 9735	13	0.1	+4	—	—	—	—
	3	0.1	—	—	—	—	-4
	4	0.1	-5(D)	—	—	—	—
♂ 9736	13	0.1	—	—	—	+3	—
	3	0.1	—	—	—	—	-1
	11	0.1	-7	—	—	—	—
	7	0.5	—	—	—	—	-10(D)
♀ 9737	13	0.1	—	—	—	-3	—
	15	0.1	—	—	—	—	-6
	7	0.5	—	—	—	—	-5(D)
♂ 9738	13	0.1	—	—	-2	—	—
	15	0.2	—	—	—	—	-14
	24	0.5	—	—	—	—	-5(D)
♀ 9739	13	0.1	—	—	-7	—	—
	7	0.2	—	—	—	—	-1
	11	0.1	±0	—	—	—	—
	13	0.5	—	—	—	—	-10(D)
♂ 9740	13	0.1	—	-4	—	—	—
	7	0.5	—	—	—	—	-9(D)
♀ 9741	13	0.1	—	+5	—	—	—
	3	0.5	—	—	—	—	-2
	24	0.1	-17(D)	—	—	—	—

dose varied from 0.1 to 1 mg. A total of 32 animals was employed, 19 females and 13 males. The experimental period lasted between 3 and 52 days.

The dietary technique employed has been recently described [Sure, 1933] by the writer. First, the animals were transferred from our stock diet No. 1 [Sure, 1926] to a ration deficient in the vitamin B complex, which consists of caseinogen (purified)¹, 20; agar-agar, 2; McCollum's salts No. 185, 4; filtered butter fat, 10; and dextrin, 64. On this ration depletion of reserves of vitamins B₁ and B₂ was secured in two and generally in not more than three weeks. The rats were then transferred to our vitamin B₁-deficient ration of the following composition: caseinogen (purified)¹, 10; McCollum's salts No. 185, 4; autoclaved dried round steak², 15; filtered butter fat, 10; dextrin, 61. On such a dietary regime, supplemented with 1 mg. daily of Sure's vitamin B concentrate [1932], a gain of 3 g. per week per animal was obtained, and with 3 to 5 mg. daily of such concentrate, a weekly gain, satisfying the Chick and Roscoe [1929] unit, was secured; yet, as much as 1 mg. daily of irradiated adenine sulphate as a source of vitamin B₁ resulted in total failure of growth. The complete results are summarised in Table I.

¹ With acidulated water and extraction with 25 % ethyl alcohol in the cold [Sure, 1933].

² Heated for 6 hours at 20 pounds pressure and dried.

SUMMARY.

Pure adenine sulphate, irradiated for periods ranging from 1 to 30 mins. and fed in doses of 0.1 to 1 mg. per rat daily proved to be a complete failure as a source of vitamin B₁ for growth.

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CCLXXIX. CRITICAL STUDY OF SHEAR'S ANILINE-HYDROCHLORIC ACID REACTION ASSOCIATED WITH VITAMIN D.

I. FURAN AND DERIVATIVES.

BY VICTOR EMANUEL LEVINE AND CHARLES LE ROY SEAMAN.

II. TERPENES.

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(Received October 28th, 1933.)

I. FURAN AND DERIVATIVES.

SHEAR [1926] reported a test associated with vitamin D made by means of a reagent containing 15 volumes of aniline and 1 volume of concentrated hydrochloric acid. When the test was positive a characteristic red coloration was obtained on heating. The test was given by irradiated cholesterol (containing ergosterol), cod-liver oil and the unsaponifiable portion of cod-liver oil, and by vegetable oils after ultra-violet irradiation.

Shear's reagent is somewhat similar to the aniline acetate reagent for detecting furfuraldehyde. We therefore undertook a study of his reagent with reference to its reactivity with furan and its derivatives. Our tests were carried out by adding 0.1 cc. to 0.2 cc. of the compound if a liquid and 25 to 50 mg. if a solid to 5 cc. of the aniline-hydrochloric acid reagent. The aniline used should be distilled once or twice until it is free from colour. We first observed the reaction at room temperature and then heated to note other changes. The results are given in Table I.

Furan and its derivatives give characteristic colour reactions with the aniline-hydrochloric acid reagent. The colours obtained in the cold vary from red, lavender, purple, green to blue. Furan, tetrahydrofuryl alcohol and tetrahydrofuryl butyrate fail to react without the use of heat. The colours obtained on heating are deep red or brown-red. The colours developed darken on standing. Furoic acid and furylacrylamide do not react at room temperature or after the application of heat.

Thiophen does not react. Pyrrole and some of its derivatives react slightly, but the reactions obtained with these compounds lose their significance in view of the fact that pyrrole when heated with dilute acid alone is known to form a reddish coloration or amorphous red powder. Pyrrole with the aniline-hydrochloric acid reagent gives a reddish brown colour before heating and a deeper brown after heating. The following compounds containing the pyrrole ring do not react in the cold or on heating: indole-3-*n*-propionic acid, skatole, proline, tryptophan and carbazole.

Table I. *Reactions of furan and derivatives with Shear's aniline-hydrochloric acid reagent.*

Compounds	At room temperature	On heating
1. Furan	No change	Deep amber with red-brown tinge
2. Furyl alcohol	Purplish red	Very dark brown
3. Furyl acetate	Brilliant red to lavender to dark lavender	Dark brown
4. Tetrahydrofuryl alcohol	No change	Orange
5. Tetrahydrofuryl acetate	Bright magenta	Deep brown-orange
6. Tetrahydrofuryl butyrate	No change	Yellow-brown
7. Furfuraldehyde	Brilliant purple	Violet precipitate with dark brown liquid
8. Furfuraldehyde acetone	Dark red, deep olive-green	Deep red
9. Furfuraldehyde acetophenone	Deep amber	Mahogany-red
10. Furylacrolein	Green to blue	Red
11. Furylacroleinoxime	Blue to green	Dark brown
12. Furfuraldehyde diacetate	Brilliant red	Mahogany-red
13. Furfuraldoxime	Bright red to purple	Dark brown
14. Hydrofuramide	Bright red to purple	Dark brown
15. Furoin	Brown, light red, dark brown with violet tint	Dark red with violet tint
16. Furil	Yellow-brown to bright red	Dark red-brown
17. Furoic acid	No change; does not dissolve	Dissolves, no change in colour
18. Methyl furoate	Orange-red	Light orange-red
19. Ethyl furoate	Blood colour, solidifies	Dark brown
20. Propyl furoate	Orange-red	Orange-red
21. <i>n</i> -Butyl furoate	Purplish red	Very deep brown
22. Amyl furoate	Deep olive-green	Orange-red
23. Furoyl chloride	Violet to light amber	Amber
24. Furonitrile	Brown-grey to dark brown	Very deep brown
25. Furaacrylic acid	Brown-grey to dark brown	Very deep brown
26. Furylacrylamide	No change	No change
27. Furylacrylyl chloride	Black-brown; does not dissolve readily	Black-brown

The Shear reagent also reacts on the application of heat when solid carbohydrates with or without a free carbonyl group are added, yielding greenish brown, yellow-brown, and brown-red colorations. The pentoses, rhamnose, xylose and arabinose, react most vigorously owing to the large amount of furfuraldehyde they are capable of yielding when heated with acid.

II. TERPENES.

Sexton [1928] studied the reagent with reference to sterols and reported negative results with cholestane, cholesteryl acetate and dicholesteryl ether. The compounds that he observed to react positively were the ketone derivatives, and of these the more unsaturated proved the more sensitive. The saturated ketones, cholestane-4-one and cholestane-7-one, proved positive after prolonged standing of the heated reaction mixture, while the unsaturated ketones, cholestenone, oxycholesterol acetate, oxycholestenone and oxycholesterylene, gave intense colour reactions within ten minutes. Two ketones belonging to the terpene series, camphor and carvone, Sexton [1928] also found to give positive reactions. His findings harmonise with those of Heilbron *et al.* [1928], who maintain that vitamin D has ketonic properties. Rosenheim and Webster [1926] regard Shear's reaction as non-specific. They were able to obtain with olive oil after the addition of a trace of benzoyl peroxide or turpentine the same intense reaction as with cod-liver oil.

We have studied Shear's reaction with members of the terpene series, using the method described in Part I (p. 2047). The results are given in Table II.

Table II. *Reactions of terpenes with aniline-hydrochloric acid reagent.*

Compound	Chemical nature	Reaction on heating	Changes on standing after heating
<i>Camphane group</i>			
1. Borneol	Saturated compound; 1 secondary alcohol group	No change	No change
2. <i>d</i> -Camphor	Saturated compound; 1 ketone group	No change	No change
3. <i>dl</i> -Camphor	"	Faint brown-red	Light brown-red
4-6. <i>d</i> -Camphoric acid <i>d</i> -Camphoric anhydride <i>d</i> -Camphorsulphonic acid	"	No change	No change
7. Fenchyl alcohol	Saturated compound; 1 secondary alcohol group	Blood-red	Brown-red
8. Pinene	Unsaturated compound; 1 double bond	Blood-red	Heavy pale brown
<i>Menthane group</i>			
9. <i>p</i> -Menthane	Saturated hydrocarbon	Brown-red	Deep red oil drops separate out
10. Cineole (eucalyptol)	Saturated compound; 1 bridge oxygen	Light brown	Darker brown
11. <i>i</i> -Menthol	Saturated compound; 1 secondary alcohol group	No change	No change
12. <i>i</i> -Menthone	Saturated compound; 1 ketone group	Dark brown	Dark brown
13. Thujone	Saturated compound; 1 ketone group	Mahogany-red	Mahogany-red
14. Terpene hydrate	Saturated compound; 2 tertiary alcohol groups	Brown-red	Brown-red
15. Terpineol	Unsaturated compound; 1 double bond and 1 tertiary alcohol group	Brown-red	Brown-red
16. Limonene	Unsaturated compound; 2 double bonds	Dark red	Darker red
17. Carvone	Unsaturated compounds; 2 double bonds and 1 ketone group	Brown-red	Deeper brown-red
18. Ionone	Unsaturated compound; 2 double bonds and 1 ketone group	Deep magenta-red; on shaking emerald green observed on sides	Darkens to deep red-brown with disappearance of green colour first observed on sides of tube when shaken
<i>Olefine group</i>			
19. Citronellaldehyde	Unsaturated compound; 1 double bond and 1 aldehyde group	Yellow	Dark brown
20. Citronellol	Unsaturated compound; 1 double bond and 1 alcohol group	Orange-red	Deep brown
21. Isoprene	Unsaturated compound; 2 double bonds	Golden yellow to yellow-brown	Brown
22. Geranial (citraldehyde)	Unsaturated compound; 2 double bonds and 1 aldehyde group	Deep cherry-red	Deep brown-red
23. Geraniol	Unsaturated compound; 2 double bonds and 1 primary alcohol group	Orange-red	Deep brown
24. Linalool	Unsaturated compound; 2 double bonds and 1 tertiary alcohol group	Dark red-brown	Deep red oil drops separate out

The compounds tested as a rule do not react in the cold. Citral, however, proved to be an exception, giving the colour of intense magenta in the cold. The colour of the reaction mixture at room temperature was slightly darker with terpene hydrate, terpineol and linalool, and slightly lighter with cineole and limonene. Citronellal gave a yellowish white precipitate in the cold, but the characteristic colour reaction was obtained on heating. The colours in the heated mixtures darkened on standing. Red colorations turned deeper red or deep brown.

The aniline-hydrochloric acid reaction is negative with *i*-menthol, borneol, *d*-camphor, *d*-camphoric acid, *d*-camphoric anhydride and *d*-camphorsulphonic

acid; *dl*-camphor reacts very faintly. The reaction is the more intense the greater the saturation of the compound. The olefinic terpenes react most vigorously. The presence of an alcohol, aldehyde or ketone group in an unsaturated terpene serves to intensify the colour reaction. Configuration also influences reactivity. Carvone and ionone are both unsaturated compounds with two double bonds and one ketone group. Yet ionone is far more reactive than carvone.

We cannot confirm Sexton's conclusion that the ketone group is responsible for the Shear reaction. The ketone, *d*-camphor, gives no result and *dl*-camphor (synthetic) gives but a very slight reaction. While the ketones, *l*-menthone, thujone, ionone and carvone, react very strongly, we must not fail to note that many of our most reactive compounds are not ketones.

Carotene yields on heating a deep brown-red colour with Shear's reagent. Carotene is related to the terpenes. Karrer *et al.* [1929; 1930; 1931] subjected β -carotene to permanganate oxidation and obtained β -ionone, a terpene. On ozonising β -carotene he recovered an amount of geronic acid comparable to that obtained from two moles of β -ionone. From α -carotene Karrer obtained amounts of geronic acid comparable with half of that obtained from β -carotene. Heilbron *et al.* [1932] obtained geronic acid as a result of the oxidation of vitamin A. The isolation of this acid confirms the presence of the ionone ring in the structure of the vitamin A molecule. We have examined ionone with the aniline-hydrochloric acid reagent and have found it to be exceedingly reactive.

The Shear reaction is given by substances containing any of the fat-soluble vitamins, by cod-liver oil and cod-liver oil concentrates, by halibut-liver oil or its concentrate and by preparations containing vitamin E. Wheat germ oil, rich in vitamin E, was treated to remove any traces of vitamin A. It was heated at 100° for one hour and oxygen passed through it during the period of heating. The cooled oil gave a stronger reaction with the aniline-hydrochloric acid reagent than the untreated oil.

The Shear reaction is also given by provitamin A (carotene) and by irradiated ergosterol.

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CCLXXX. REACTIONS OF TERPENES WITH ANTIMONY TRICHLORIDE.

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(Received October 28th, 1933.)

ANTIMONY trichloride in chloroform solution yields colour reactions with fish-liver oils and fish-liver oil concentrates [Carr and Price, 1926], with carotenoid pigments [Moore, 1929; Euler *et al.* 1928], with certain sterols and their derivatives [Karrer, 1928; Wokes, 1928; Heilbron and Spring, 1930; Seel, 1931], and with certain five-membered monoheterocyclic compounds [Levine and Richman, 1933].

Unsaturation and the terpene groupings are common chemical factors in carotenoid pigments, in vitamin A and in sterols occurring in the plant and animal organism, according to Karrer *et al.* [1929; 1930; 1931] and to Heilbron, Morton and Webster [1932]. Norris and Church [1930] have studied the antimony trichloride reaction with relation to some thirty-four essential oils. They reported that these oils yield various shades of yellow, brown and red. Wormwood oil, however, gave a green colour, etheral oil a purple and cedar wood oil an intense permanent blue, which has an absorption band with a maximum at $580 m\mu$. Corbet *et al.* [1933] reported several terpenes as interfering substances in the antimony trichloride test for vitamin A in cod-liver oil.

We have studied the antimony trichloride reaction among compounds of the terpene group. The reagent used was that of Carr and Price. Antimony trichloride, washed three times in chloroform (U.S.P.) containing 1 % alcohol, was dried in the desiccator. A solution of this product was made by adding 30 g. to 100 cc. of chloroform. The clear solution was decanted and used as the reagent. The terpenes were made up to 20 % solutions or mixtures in chloroform. Three to four drops of the chloroform mixture were added to 2 cc. of the antimony trichloride reagent. The reaction mixture containing terpene and reagent we have designated as Reaction mixture A.

We have also adopted a procedure in which three to four drops of the terpene in chloroform are added to 0.5 cc. of acetic anhydride before final admixture with antimony trichloride solution. The acetic anhydride prevents the formation of precipitates in the reaction mixture. It also acts as a catalytic agent, inducing a more vigorous reaction and a more intense display of colour changes. The reaction mixture containing acetic anhydride we have designated as Reaction mixture B. The results are given in Table I.

The reaction is usually characterised by a succession of colour changes. In many cases the final colour is purple. Citral in the presence of acetic anhydride changes from dark yellow to brown, to wine, to purple and finally to dark blue. Ionone is a terpene which may be obtained by the oxidation of carotene and of vitamin A [Karrer *et al.*, 1929; 1930; 1931]. This unsaturated terpene gives with antimony trichloride an amber colour changing to wine, and finally on long

Table I. *Reaction of terpenes with antimony trichloride.*

Compound	Chemical nature	Reaction mixture	Results
<i>Camphane group</i>			
1. Borneol	Saturated compound; 1 secondary alcohol group	A	Colourless, slight pink overnight
		B	Colourless; no change
2. <i>d</i> -Camphor	Saturated compound; 1 ketone group	A	Light pink to rose
		B	Colourless, to amber with pink tint
3. <i>d</i> -Camphor	"	A	No colour after 24 hours
		B	No colour after 24 hours
4-7. <i>d</i> -Camphoric acid	"	A	No colour after 24 hours
<i>d</i> -Camphoric anhydride		B	No colour after 24 hours
<i>d</i> -Camphorsulphonic acid			
8. Fenchyl alcohol	Saturated compound; 1 secondary alcohol group	A	Deep lemon turning cloudy green, finally white precipitate and light brown fluid
		B	Colourless, changing quickly to deep lemon-yellow, brownish yellow, purple and to wine colour with brown tinge
9. Pinene	Unsaturated compound; 1 double bond	A	Creamy, chalky precipitate, to orange precipitate and brown wine-coloured liquid
		B	Colourless, then brown-purple colour, finally light brown
<i>Menthane group</i>			
10. <i>p</i> -Menthane	Saturated hydrocarbon	A	Slightly cloudy, light pink, to salmon colour, gray precipitate
		B	Colourless, to light lemon, light amber and finally light red-brown
11. Cineole (eucalyptol)	Saturated hydrocarbon; 1 bridge oxygen	A	Cloudy with pink tint. On standing precipitate forms, supernatant liquid orange with purple tint then to purple-brown
		B	Colourless to pink, purple-pink, rose-purple to purple
12. <i>l</i> -Menthone	Saturated compound; 1 ketone group	A	Colourless; cloudy faint pink after 24 hours
		B	Pale straw, to very light tan after 24 hours
13. <i>l</i> -Menthol	Saturated compound; 1 secondary alcohol group	A	No colour
		B	No colour
14. Thujone	Saturated compound; 1 ketone group	A	Slightly cloudy, to light pink, yellow pink, to brown with pink tinge
		B	Slight amber, dark amber, brown
15. Terpene hydrate	Saturated compound; 2 tertiary alcohol groups	A	Cloudy, light orange with pink tinge, overnight light brown with pink tint
		B	Colourless, to pink, overnight to mahogany-brown with pink tint
16. Terpineol	Unsaturated compound; 1 double bond and 1 tertiary alcohol group	A	Light liquid, then pinkish precipitate and orange-yellow liquid, which becomes purple-brown, finally brown with slight green tinge
		B	Colourless, to lemon colour, light pinkish brown, reddish brown
17. Limonene	Unsaturated compound; 2 double bonds	A	Creamy suspension, to brick colour; white precipitate with a brown liquid, which finally changes to purplish
		B	Greenish lemon, to mahogany-brown and finally to purplish brown
18. Carvone	Unsaturated compound; 2 double bonds and 1 ketone group	A	Lemon yellow changing to rose
		B	Lemon yellow changing to red-brown
19. Ionone	Unsaturated compound; 2 double bonds and 1 ketone group	A	Amber to wine, overnight to purple
		B	Amber to greenish amber, mahogany-brown to reddish purple

REACTIONS OF TERPENES WITH ANTIMONY TRICHLORIDE 2053

Table I (cont.).

Compound	Chemical nature	Reaction mixture	Results
<i>Olefine group</i>			
20. Citronellal	Unsaturated compound; 1 double bond and 1 aldehyde group	A	White flaky precipitate, to orange, to pink supernatant red-brown fluid
		B	Amber, purple amber, brownish purple to wine colour
21. Citronellol	Unsaturated compound; 1 double bond and 1 primary group	A	Yellowish suspension, cloudy brown, to brown, to orange, to red-brown
		B	Amber, purple amber, brownish purple to wine colour
22. Isoprene	Unsaturated hydrocarbon; 2 double bonds	A	Yellow at once, changing to orange and then to cherry-red liquid with brown precipitate
		B	No colour formation within first half hour, then yellow to orange, to deep red, to brown
23. Geraniol	Unsaturated compound; 2 double bonds and 1 primary alcohol group	A	Creamy coloured suspension, to orange, brown-orange, mahogany-brown
		B	Lemon, changing instantaneously to faint pink, then pink-purple, to purplish brown
24. Geranial (citral)	Unsaturated compound; 2 double bonds and 1 aldehyde group	A	Dark yellow precipitate, dark brown liquid
		B	Dark yellow, brown brick precipitate, mahogany-brown liquid changing to purple tint
25. Geranyl acetate	"	A	Golden yellow, brownish brick precipitate and mahogany-brown liquid to purple tint
		B	Greenish lemon, light green, light gray-brown with purple tint
26. Linalool	Unsaturated compound; 2 double bonds and 1 tertiary alcohol group	A	Light orange suspension, light pink, yellow-pink, brown with pink tinge
		B	Lemon, turning instantaneously to light purple-brown, purple mahogany-brown, to distinct purple-brown

standing to purple. In the presence of acetic anhydride the initial colour is amber, which changes to greenish amber, mahogany-brown and finally to reddish purple. Vogel and Stohl [1933] converted β -ionone by cyclisation with sulphuric acid into an orange-yellow powder, which gave with antimony trichloride in chloroform solution a permanent and very intense blue colour with a maximum absorption spectrum at $510m\mu$ and a large band beginning at $430m\mu$.

The following compounds yield no colour reactions: *dl*-camphor, *d*-camphoric acid, *d*-camphoric anhydride, *d*-camphorsulphonic acid and *l*-menthol. The following react very slowly and very slightly: *d*-camphor, borneol and *l*-menthone.

The terpenes that react most vigorously are the unsaturated ones and the higher the degree of unsaturation, the greater the reactivity of the compound. The presence of an aldehyde, alcohol or ketone group induces greater reactivity in the unsaturated compound. The camphane group is the least reactive and the olefine group the most reactive.

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CCLXXXI. CEREALS AND RICKETS.

IV. THE EFFECT OF IMMATURITY OF THE MAIZE KERNEL UPON ITS RACHITOGENIC PROPERTIES¹.

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(Received October 7th, 1933.)

INTRODUCTION.

THE present report on the effect of the ripening process on the anticalcifying properties of maize had its origin in two lines of interest. In the first place, it was expected that if differences between immature and ripe grains were found to exist, the foundation might thereby be laid for profitable studies on the nature of an anticalcifying factor or factors [Mellanby, 1921; 1922; 1924; 1925; 1926; 1930; Green and Mellanby, 1928]. In the second place, the irregularity in the production of rickets on Ration 2965 [Steenbock and Black, 1925] as reported by Harris and Bunker [1930; 1931] might be explained. In seven or more years of use of Ration 2965, even though we have obtained our supply of maize from numerous sources on the open market, we have not experienced the irregularities in the production of rickets reported by Harris and Bunker. They claim to have overcome the difficulty by ageing the ground maize for 6 months or more, but storage of the whole kernels as distinguished from the ground maize was not found to result in improvement. Goldblatt [1931] has suggested that the difficulty was not caused by a variation in the quality of the maize but in a variable settling out of the calcium carbonate from the other ingredients of the ration. He has proposed the incorporation of gelatin in the diet to prevent this.

EXPERIMENTAL.

Series I and II—Field maize.

On August 25, 1931, yellow dent maize still in the milk stage was brought to the laboratory from the university farm. Only good sized ears which had the husks fully closed and which carried a browning silk were selected. These were husked in the laboratory, and the kernels were cut from the cob. Scraping successfully removed the germ from the cob also. A moisture determination at this time showed the presence of 66.0 % of water.

The maize, spread in granite-ware pans in a layer not over one kernel in depth, was dried in the laboratory before an electric fan at a temperature not exceeding 29°. If any fermentation occurred, it was kept at a minimum by the low humidity of the atmosphere during the days of drying. The product had a sweet odour and in 24 hours was sufficiently brittle to be ground in a burr mill.

¹ Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

² Quaker Oats Company Fellow.

The temperature during grinding never rose above 42°. Immediate cooling was accomplished in a refrigerator. After a day of further desiccation at reduced pressure in the presence of dry CaCl_2 , it was stored in a cloth bag.

A second portion of the maize prepared in the same manner was desiccated in a drying room with the aid of a fan. After 15 hours' drying at a temperature slightly below 50° it was sufficiently dry to grind. A third portion, which was too mature to be classified in the milk stage, was dried on the cob in an air current at room temperature preparatory to shelling and grinding.

For control purposes normally matured ears of maize were removed from the shocks of the same field on September 29. These were shelled and dried by exposure to an air current at room temperature for 5 days. The kernels were ground at once and feeding was started a week later. For additional control purposes there was fed maize from the previous year's harvest.

In Series I each sample of maize was fed as the cereal component of our high calcium-low phosphorus rickets-producing Ration 2965 [Steenbock and Black, 1925] which has the following composition: yellow maize 76 parts, wheat gluten 20; CaCO_3 3; and NaCl 1. The rations were fed to four litters of rats ranging in age from 23 to 25 days and in weight from 54 to 67 g. The rats were confined individually in cages provided with screen bottoms. They were so distributed that each ration was fed to one representative of a litter. Distilled water and food were fed *ad libitum*, but a daily record was kept of the food consumed. Body weights were recorded weekly.

In Series II there was used the same maize as in Series I, but the ration and technique of feeding were changed. Four parts of the wheat gluten were replaced by dry yeast¹ to improve food consumption, and the food intake was equalised by limiting it for each rat in the series to the level of that rat which had the lowest intake, disregarding distinctly abnormal animals. Each ration was fed to 6 rats taken from representative litters at the age of 25 or 26 days and at a range in weight of 52 to 63 g.

At the close of a 5-week feeding period the rats of both Series I and II were killed and the femurs removed and dissected free from soft tissues. The femurs were extracted in a Soxhlet extractor for five days with 95 % alcohol and then ashed in a muffle furnace at red heat. The distal ends of radii and ulnae were preserved in 10 % formalin and later split longitudinally and placed in dilute AgNO_3 solution for exposure to light to reveal the width of the uncalcified metaphyses as carried out in the line test described by McCollum *et al.* [1922]. Inspections of the costochondral junctions were made at the time of termination of the experiment.

The average results for the groups studied in Series I and II are assembled in Table I. In the series which received the rations *ad libitum* but without yeast, the range of average individual daily food intake was 6.2 to 9.1 g. with group averages ranging from 7.0 to 8.2 g. In the duplicate series of rations containing yeast, the individual range was 6.5 to 7.3 g. with group averages between 6.9 and 7.2 g.

The weight gains as well as food consumption of the rats were somewhat disturbed during the fifth week by infections of their respiratory tracts. Although in most cases the infections were of a very mild nature, in 10 animals they were of a moderate or severe grade as indicated by a definite wheeze and the presence of a serous discharge from the nostrils. Although these symptoms were suggestive of a vitamin A deficiency, only two rats showed evidence of the initial stages of ophthalmia.

¹ Obtained from The Northwestern Yeast Company, Chicago.

Table I. *Effect of immaturity of field maize on calcification.*

Ration	Body weight		Average daily food consumption g.	Femur data			Meta-physes	Costochondral junctions
	Initial g.	Gain g.		Weight of ex-tracted bone g.	Weight of ash g.	% of ash		
	Ad libitum food consumption—(4 animals)							
R 37. Unripe maize air-dried below 29°	58	42	7.9	0.1005	0.0331	33.0	Wide	Much enlarged; double beading; angulation
R 38. Unripe maize dried at 50°	59	46	7.9	0.1069	0.0346	32.3	„	„
R 39. Nearly ripe maize air-dried on cob	60	18*	8.2	0.0833	0.0203	24.3	„	„
R 40. Ripe maize, from shock	58	17*	7.2	0.0893	0.0250	27.8	„	„
R 41. Ripe maize, from usual stock supply	61	24	7.0	0.0929	0.0243	26.2	„	„
Equalised food consumption—(6 animals)								
R 42. Unripe maize air-dried below 29°	57	44	7.2	0.1050	0.0330	31.5	„	„
R 43. Unripe maize dried at 50°	57	44	7.2	0.1069	0.0357	33.2	„	„
R 44. Nearly ripe maize air-dried on cob	(55)	(25)	(7.0)	(0.0840)	(0.0213)	(25.5)	„	„
	57	24*†	7.0	0.0902†	0.0243†	26.8†	„	„
R 45. Ripe maize, from shock	55	33*	6.9	0.0924	0.0220	24.0	„	„
R 46. Ripe maize, from usual stock supply	56	30*	7.0	0.0903	0.0210	23.2	„	„

* 2 or 3 animals lost weight during the last week.

† 3 animals died before 31st day.

() Average for the 3 animals which lived to the close of the experiment.

The values for weights of extracted femurs, weights of ash and percentages of ash ran remarkably parallel in the two series. Although all animals were in a very rachitic condition, the ash analyses indicated that the immature maize was less rickets-producing than the ripe maize. Group averages for percentage of ash in the femurs of rats fed immature maize, whether dried below 29° or at approximately 50°, fell between the values 31.5 and 33.2 %. On the other hand the somewhat immature maize dried on the cob, ripe maize taken from the shock and maize from the previous year's stock supply produced femurs with an ash content falling between 23.2 and 27.8 %. Thus immature maize rations consistently produced a bone with approximately 7 % higher ash content than that formed from ripe maize rations. The average weight of extracted femurs from the immature maize rations fell between 0.1005 and 0.1069 g. as compared with the ripe maize range of 0.0833 to 0.0929 g. A similar relationship held for average ash weights, the immature maize values falling between 0.0330 and 0.0357 g. in comparison with a range of 0.0203 to 0.0250 g. for the ripe maize products.

The silver nitrate staining test as applied to the wrist bones showed the presence of wide rachitic metaphyses in all animals of both series. Likewise no differentiation was possible by means of the costochondral junctions, all of which were much enlarged with double beading or angulation in most animals. These observations were in harmony with the results obtained by ash analysis of the femurs.

It is evident that the immature maize contained nutrients or a combination thereof, which promoted better bone calcification than the corresponding mature maize. Analysis of two of the rations of Series I ruled out the possibility that this was due to a variation in total phosphorus content. The ration compounded from immature maize dried below 29° contained 378 mg. of phosphorus per 100 g., and that compounded from ripe maize from the shock contained 391 mg. per 100 g.

Series III—Sweet maize.

The study of the relation between the ripening process of maize and a change in its power to promote bone calcification was continued on canned sweet maize. Two canned products of "Golden Bantam" sweet maize and "Golden Bantam" seed were obtained, from a commercial canning company¹. One product was vacuum-packed and the other brine-packed. According to information furnished by the packers the sweet maize canned by the vacuum process had received no addition other than salt. In the brine process of packing, salt, sugar and water had been added. For further comparison a brine-packed whole kernel white sweet maize, probably of the "Country Gentleman" variety, was bought in the open market.

The desiccation of the above canned products was carried out in granite-ware pans placed before a fan in a room held at a temperature of 47 to 51°. By spreading the maize in a layer not over one kernel in thickness a product suitable for grinding was obtained in 18 to 22 hours. The vacuum-packed maize yielded 31 % of dry matter and the brine-packed maize an average of 20 % dry matter.

The three samples of canned sweet maize were compared with ripened Golden Bantam seed. In order to relate the results to those obtained in the immediately preceding series, immature yellow dent maize dried below 29° was also included. The possible effect of heating during the drying process was evaluated by using a ration which included maize which had been ground coarsely, then covered with water and dried under conditions comparable to those used in desiccating the canned samples.

The 6 samples of maize were fed in Ration 2965 which was modified by the inclusion of 4 % dry yeast as described in Series II. To eliminate a possible vitamin A deficiency indicated in the previous experiments, 2 drops of red palm oil were administered to each rat twice weekly. This was five times the amount required to cure ophthalmia in rats depleted of vitamin A.

Before feeding the red palm oil as a source of vitamin A, it was tested for vitamin D to make certain that it did not add appreciable amounts of this factor. The assay was carried out in the usual manner of conducting the line test. Rachitic rats were fed the oil at levels of 2, 5 and 7 drops, delivered from a calibrated dropper. These amounts, equivalent to 40, 100 and 140 mg. of oil, were administered over a 10 day period. In no case was any evidence of healing obtained in the distal ends of the radii and ulnae.

Each ration was fed to 6 rats taken from litters 24 to 27 days of age. The distribution, caging and feeding of the animals were the same as in Series II, including equalisation of food consumption for all rats.

At the close of the 5 week feeding period pooled blood samples were obtained for estimation of serum-calcium and inorganic phosphorus. The rats were placed under light ether anaesthesia and the blood from the left carotid artery was collected in a chilled centrifuge-tube. After standing overnight in a refrigerator, the blood was centrifuged and the serum was analysed.

The inorganic phosphorus was determined by the method of Fiske and Subbarow [1925]. Calcium was determined by the McCrudden [1911-12] method modified as follows. An aliquot of the protein-free filtrate obtained as in the phosphorus determination and representing 1 cc. of serum was brought to neutrality in the presence of alizarin with dilute NH_4OH and HCl . One drop of 5 % HCl in excess was added and the calcium precipitated by sodium oxalate in

¹ Obtained from The Mid West Canning Corporation, Rochelle, Illinois.

RACHITOGENIC POTENCY OF YOUNG AND OLD MAIZE 2059

a medium buffered with sodium acetate to a p_H sufficient to give a red colour with alizarin. After standing overnight, the calcium oxalate was centrifuged off, washed with dilute NH_4OH , dissolved in H_2SO_4 and titrated with 0.01 N $KMnO_4$ delivered from a micro-burette.

The ash analyses were made on only one femur of each animal except in those cases where a possible discrepancy needed checking. The metaphyses of the wrists were measured after silver nitrate staining, with a steel scale graduated to one-hundredth of a centimetre. Observations on the costochondral junctions were included.

The results of this series of tests are assembled in Table II. The gains in body weight were both satisfactory and uniform throughout all groups. The food intake averaged 6.6 g. per animal per day for the entire period.

Table II. *Effect of immaturity of sweet maize on calcification.*

Ration	Body weight		Average daily food consumption g.	Blood-serum analysis*		Femurs			Maximum width of metaphyses cm.	Costochondral junctions
	Initial g.	Gain g.		Ca mg./100 cc.	P mg./100 cc.	Weight of extracted bone g.	Weight of ash g.	% of ash		
R 61. Golden Bantam seed	63	47	6.6	11.4	2.5	0.1138	0.0378	33.0	0.15	Much enlarged, some angulation and double beading
R 62. Golden Bantam ground seed moistened and dried	62	52	6.6	10.8	2.7	0.1174	0.0424	36.2	0.14	Much enlarged
R 63. Golden Bantam, vacuum pack	62	51	6.6	11.0	4.3	0.1450	0.0653	45.0	0.10	Slightly enlarged
R 64. Golden Bantam, brine pack	60	54	6.6	11.2	3.9	0.1419	0.0619	43.6	0.10	Slight to moderately enlarged
R 65. White sweet maize, brine pack	62	57	6.6	12.0	4.4	0.1620	0.0749	46.1	0.10	Slightly enlarged
R 66. Unripe maize, air-dried below 29° (yellow dent)	61	55†	6.6	10.8	3.9	0.1338	0.0534	39.7	0.11	Moderately enlarged

* Blood from rats with severe respiratory trouble was not used.

† One rat had severe respiratory trouble at the close of the experiment.

The percentage of ash in the femurs disclosed the same relations as those observed in the previous experiments; that is, the immature maize promoted better calcification than the corresponding ripe maize. Whereas the Golden Bantam seed produced femurs containing 33.0 % of ash, the same variety of maize at the canning stage raised the ash content to 45.0 and 43.6 %. The white variety of sweet maize gave an ash content of 46.1 %, a figure comparable to that given by the Golden Bantam variety. Moistening and redrying the Golden Bantam seed raised the ash content to 36.2 %—a 3.2 % increase above the untreated seed. This suggested the need for further study of the complicating factors of moistening and heating. The 39.7 % of ash in the bones produced on immature yellow dent maize dried below 29° fell between the percentages obtained for the immature and ripe sweet maize. It was also 7 to 8 % higher than the figure obtained for the same sample used in the previous tests. This latter variation can be accounted for by such factors as difference in initial weight of animals and food consumption.

These differences in calcifying properties were verified by the analyses of the blood-sera. Whereas all calcium values fell within the normal range, the inorganic phosphorus was greatly depressed. Although it was impossible to obtain

particularly accurate results at the low levels encountered, the relative distribution of values was the same as in the data already reviewed.

Another confirmation of these relations was observed in the width of the rachitic metaphyses of the wrist bones and in the degree of involvement of the costochondral junctions.

Since any variations in calcification might be caused by differing phosphorus content of the rations, the dried maize samples were analysed for this element using the method described by The Association of Official Agricultural Chemists [1930]. The three samples of canned sweet maize were found to contain between 264 and 273 mg. phosphorus per 100 g. dry product, the Golden Bantam seed 355 and 367 mg. and the immature yellow dent maize 410 mg. From these differences calculations based on a daily food intake of 7 g. indicated that the maximum variation in total phosphorus intake over a 5 weeks' period was 272 mg. It is obvious that these phosphorus variations were not responsible for the observed differences in calcification since the sweet maize rations with the lower phosphorus content produced better calcified bones.

Sweet maize at the canning stage possessed properties favouring better skeletal calcification than the same maize fed as ripened seed. This is in accord with the previous results obtained with immature yellow dent field maize.

SUMMARY.

Immature yellow dent field maize promoted better calcification than corresponding mature maize of the same variety and grown under identical conditions.

Commercially canned sweet maize promoted better calcification than the seed from which it had been grown.

The differences in calcifying value cannot be explained by more favourable calcium/phosphorus ratios.

The authors wish to express their appreciation to the Quaker Oats Company whose financial support made these experiments possible. The authors are indebted to Mr James T. Lowe and Mr Robert W. Haman for assistance rendered in analytical work.

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CCLXXXII. CEREALS AND RICKETS.
V. THE EFFECT OF GERMINATION AND AUTOLYSIS
ON THE RACHITOGENIC PROPERTIES OF
THE MAIZE KERNEL¹.

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(Received October 7th, 1933.)

INTEREST has been centred not only on the effect of maturity on the calcifying properties of grains [Templin and Steenbock, 1933] but also on changes effected by the process of germination. Stepp [1925] observed that the substitution of sprouted grains for the cereal component of McCollum's rachitogenic ration 3143 [McCollum *et al.*, 1921] did not alter its rickets-producing effect.

Mellanby [1926] reported that the rachitogenic property of oats was not materially altered by exposure to a temperature of 100° for 18 hours or by germination for as long as a week, but the combined application of these treatments distinctly reduced interference with bone calcification. He further stated that the same relations held for barley, making the kilned grain a comparatively good cereal product from the point of view of bone formation and much better than the original barley.

György and Schall [1929] extended the study of the rickets-producing property of seeds germinated in the dark to include wheat, barley, oats and beans (Puffbohnen). He used extracts of these germinated seeds prepared with a mixture of alcohol, ether and chloroform of such concentration that 0.1 g. of extract represented 1 g. of seed. No evidence of healing was obtained when daily doses of 0.1 g. of extract were administered to rachitic rats over a period of 8 to 14 days. These workers were also unsuccessful in their attempt to activate ergosterol by exposing it to mitogenetic rays from the roots of hyacinth bulbs.

The observations of Schittenhelm and his co-workers stand in opposition to the results just reviewed. Rubner and Schittenhelm [1926] expressed interest in the promotion of the therapeutic use of preparations made from dried sprouts obtained as a by-product of barley malted in the dark. Schittenhelm and Eisler [1928, 1] claim to have demonstrated the presence of vitamin D in these sprouts by feeding rats with an extract obtained with alcohol, light petroleum and chloroform. They found that 1 or 2 mg. of extract daily were sufficient to induce healing in 8 days. They further state that the active principle follows the phytosterol fraction and is sensitive to oxygen and to light. However, the incompleteness of the data reported as well as the inadequate use of controls make it difficult properly to evaluate these results.

¹ Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

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At a later date these same investigators [1931, 2, 3, 4] reported more carefully controlled experiments strengthening their claim that antirachitic activity is present in dried barley sprouts. In prophylactic tests, when dried sprouts comprised 6 % of the otherwise rachitogenic ration, rickets was prevented, and bone with a normal ash content was formed. Their failure to do more than arrest further development of rickets by feeding the same dried sprouts at even higher levels to rachitic rats they attribute to deranged functions of the digestive tract occurring in rats suffering with rickets. Analysis revealed that twice as much crude fibre was eliminated by the rachitic animals as by normal rats of a similar age. Because of this complication, they found it more satisfactory to work with an extract prepared with a mixture of acetone and light petroleum. Daily doses of 1 mg. of the extract were sufficient to prevent or cure rickets consistently in rats and further fractionation resulted in even more potent products.

Rubner [1930] has reported improvement in the retention of nitrogen, calcium and phosphorus in two patients who were given 50 g. of dried barley sprouts daily; but these results were complicated by the feeding of a bone-marrow supplement.

On the basis of such findings together with other clinical data, Schittenhelm [1928] and in collaboration with Eisler [1928, 2; 1931, 1] has advanced the thesis that the formation of vitamin D is not dependent upon the presence of ergosterol or activation by light. They favour the point of view that it is formed during the process of germination, even in the dark, since they have failed to detect provitamin in the seed from which their preparations were made.

Our objective in the present series of studies was to determine if any alteration in the rickets-producing property of maize occurred during germination in the absence of light. We relied primarily upon prophylactic technique in which the products tested were substituted for the cereal component of a high calcium-low phosphorus rachitogenic ration. A few curative tests were conducted with alcoholic extracts, but the data accumulated are as yet too limited to warrant presentation.

EXPERIMENTAL.

Series I. In order to correlate results with those already published [Templin and Steenbock, 1933], the maize selected for these experiments was of the same origin and was raised in the same field and harvested and stored according to the usual agricultural practice.

The maize was germinated in the following manner. A weighed quantity was thoroughly scrubbed with three changes of distilled water and then treated in a vacuum with a 1.0 % solution of formalin for 10 minutes. After an additional 10 minutes at atmospheric pressure the formalin was drained off, the maize was washed once or twice with sterile water and finally covered and allowed to soak in sterile water from one and a half to six hours. Tinned or galvanised iron pans 12 × 20 × 25 inches were used as germinators. In them was placed a thin layer of clean pine shavings which was covered with two layers of paper towelling. A litre of water was poured in, the pan was wrapped with heavy manila paper and sterilised for 15 minutes in an autoclave at 15 pounds pressure. Approximately 250 to 300 g. of prepared maize were then spread in a single layer between the towelling. Care was exercised to reduce contamination to a minimum. The germinator was incubated at 26°. In the longer germination periods it was found necessary to add more sterile water later. In 48 hours 95 % of the kernels had grown roots not exceeding 1 inch in length and 5 % had roots between 1 and 2 inches in length. In 95 hours only 23 % of the roots were less than 1 inch in length, 65 % between 1 and 3 inches, and 12 % between 3 and 4 inches.

At the termination of the germination period, the maize was ground in a food chopper. A portion was spread in granite-ware pans in a layer not over $\frac{1}{2}$ inch deep and dried before a fan at a temperature of 35 to 37° for 24 to 48 hours. The dry product was ball-milled for incorporation in the test ration.

For autolysis a second portion of the sprouted maize was ground, and approximately 200 g. quantities were placed immediately in 2 quart glass jars. One litre of distilled water, with chloroform and toluene, was added; the cover was lightly closed, and the whole was placed in an incubator for 10 days at 31°. The product was dried in the same manner as the germinated preparation. Difficulty was encountered in desiccating the autolysed corn which had been germinated 95 hours because enough sugars were present to form sticky lumps which failed to release all moisture and toluene even when placed in a CaCl_2 desiccator provided with a circulating air current. Final desiccation was attained in an evacuated desiccator kept at 45°. In all it required about two weeks to dry this product sufficiently to allow grinding in a burr mill preparatory to incorporation in the ration. A 90 % recovery of the initial weight of the maize was obtained in the products of maize germinated 48 hours and an 83 % recovery in the case of maize germinated 95 hours.

The dry, ball-milled maize products were substituted for the yellow maize in Ration 2965 [Steenbock and Black, 1925] with the further addition of 4 % yeast known to be rich in the vitamin B complex. This was added at the expense of the wheat gluten fraction.

Seven litters of rats between 24 and 27 days of age were used to provide 6 rats for each test ration. The daily food intake of each rat was restricted to that amount voluntarily taken by the rats with the lowest food consumption. The limiting individuals of the entire series were rats receiving the maize which had been sprouted 95 hours and autolysed 10 days. During the first three weeks approximately three times the requirement of vitamin A was administered to each rat weekly in the form of a crude carotene extract dissolved in corn oil. During the last two weeks a similar amount of vitamin A was fed as red palm oil. Data on the composition of the bones and blood were obtained as described in the preceding publication [Templin and Steenbock, 1933].

Data. The results of this series of experiments are to be found in Table I. It will be noted that weight gains and food consumption were both satisfactory and uniform throughout the various groups. Distinctly the best calcification was obtained with the rations which contained autolysed sprouted maize. Whereas untreated maize and maize sprouted for 48 and 95 hours respectively gave 27.9, 28.4 and 29.6 % of ash, maize sprouted for 48 and 95 hours and then autolysed raised these figures to 46.6 and 56.7 respectively. Thus the sprouting process altered the calcifying activity only to a very slight degree if at all, but when followed by autolysis practically normal bone was produced. Soaking the maize for 16 hours increased the ash content to 35.5 %, but in a later test this result could not be duplicated.

Criteria other than percentage variation in bone ash confirmed the indicated relations. Whereas the blood-serum-calcium was normal, the inorganic phosphorus was very low in all groups except the one receiving maize which had been sprouted 95 hours and autolysed 10 days. This group had a normal value of 7.8 mg. per 100 cc. The width of the rachitic metaphyses and to a lesser extent the enlargement of the costochondral junctions also confirmed these relations.

From the results of this series of experiments it appears that maize germinated in the dark for as long as 4 days does not acquire additional antirachitic

Table I. *Effect of germination and autolysis of maize upon calcification.*

Ration	Body weight		Average daily food consumption g.	Blood-serum analyses*		Femur data			Maximum width of metaphyses cm.	Costochondral junctions
	Initial g.	Gain g.		Ca mg./100 cc.	P mg./100 cc.	Weight of extracted bone g.	Weight of ash g.	% of ash		
R 47. Untreated	57	33	7.0	12.4	2.7	0.0976	0.0277	27.9	0.17	Much enlarged, angulation, double beading
R 48. Soaked 16 hours	56	34	7.0	13.2	4.6	0.1094	0.0393	35.5	0.17	Moderately to severely enlarged, angulation and double beading in 2 animals
R 49. Soaked and sprouted totalling 48 hours	57	38	7.0	13.2	3.3	0.1024	0.0293	28.4	0.17	Much enlarged, angulation, double beading
R 50. Soaked and sprouted totalling 48 hours, autolysed 10 days	56	36	7.0	14.0	4.5	0.1307	0.0615	46.6	0.08	Slightly to moderately enlarged
R 51. Soaked and sprouted totalling 95 hours	57	24†	6.9†	11.6	2.7	0.0900	0.0270	29.6	0.15	Much enlarged, angulation, double beading
R 52. Soaked and sprouted totalling 95 hours, autolysed 10 days	57	32	7.0	13.2	7.8	0.1751	0.0977	56.7	0.05	Normal

* Blood of animals having advanced respiratory trouble was not used.

† 2 animals had severe respiratory trouble.

potency. On the other hand autolysis of this germinated maize results in changes which materially aid calcification. However, the effect of heat as a complicating factor was not completely ruled out.

Series II. To determine the part which heat and moisture conditions played in the previous series, the following group of maize rations was fed: (1) germinated 96 hours and autolysed 10 days; (2) germinated 96 hours—stored dry at same temperature as (1); (3) germinated 96 hours—boiled—stored wet at same temperature as (1); (4) soaked 16 hours and dried for 24 hours; (5) ground and dried for 24 hours; (6) untreated.

The maize used was a high grade yellow dent seed maize grown in Wisconsin. The process of germination was conducted as previously described except for the elimination of the soaking before germination and the use of a larger amount of maize, *viz.* 400 g. per pan. Germination proceeded vigorously at 31°. In 96 hours 45 % of the kernels had rootlets 3 to 5 inches in length, 45 % 1 to 2 inches and 10 % less than 1 inch. Autolysis was conducted at 31° using CHCl_3 as an antiseptic. By an increase in the temperature of drying to a range of 45 to 49°, it was possible to shorten the time of drying of the autolysed product to 6 days when carried out in open pans before an electric fan. Controls for such heat treatment were provided by the following two preparations. Dried germinated maize was stored alongside the autolysed product both during its autolysis and drying. The other control was germinated maize which had been boiled 5 minutes to inactivate the enzymes, then stored and dried by the methods used in the preparation of the autolysed product. Other groups were included in the series to check the result on the effect of soaking the whole kernels and to determine the result of heat treatment on untreated ground maize.

The prepared samples were fed in Ration 2965 according to the technique described for the preceding series. During the first 13 days of feeding the average daily intake of ration per rat was only 4.5 g. owing to the fact that the rats did not like the autolysed and boiled maize rations. Since this amount was not sufficient to maintain good growth, it was decided to replace only half of the

yellow maize of the ration with these products for the remainder of the 5-week period. This necessitated a similar change in the ration of germinated maize which had been stored under dry conditions. Such an alteration in the dietary regimen resulted in a 7 to 8 g. daily intake per rat and a resumption of satisfactory growth. Calculations in terms of the total ration eaten over the entire experimental period indicate that these prepared products comprised 49 % of the ingested food.

The average results of the data obtained by the same methods employed in Series I are to be found in Table II. Body weight gains of 30 to 40 g. were procured on an average daily food intake of 6.0 g. Such gains were comparable with those obtained in preceding tests.

Table II. *Effect of germination, autolysis and heat treatment of maize upon calcification.*

Ration	Body weight		Average daily food consumption g.	Blood-serum analyses		Femur data			Maximum width of metaphyses cm.	Costochondral junctions
	Initial g.	Gain g.		Ca mg./100 cc.	P mg./100 cc.	Weight of extracted bone g.	Weight of ash g.	% of ash		
R 72. Germinated 96 hours and autolysed 10 days	57	30	6.0	13.0	3.4	0.1149	0.0540	46.8	0.06	Slightly enlarged
R 73. Germinated 96 hours—stored dry at same temperature as R 72	58	32	6.0	11.5	3.0	0.0901	0.0265	29.3	0.15	Much enlarged with double beading and angulation
R 74. Germinated 96 hours—boiled, stored wet at same temperature as R 72	59	29	5.9	13.1	4.0	0.1056	0.0419	39.3	0.12	Moderately to severely enlarged
R 75. Soaked 16 hours, dried 24 hours	58	37	6.1	12.2	3.3	0.0997	0.0333	33.3	0.15	Much enlarged
R 76. Ground, dried 24 hours	59	41	6.1	11.6	3.5	0.1070	0.0343	31.7	0.14	Much enlarged
R 77. Untreated	57	39	6.1	12.6	4.0	0.1033	0.0364	35.2	0.13	Much enlarged

The blood-serum picture was as expected, namely, a normal range of calcium and a greatly depressed inorganic phosphorus content without significant variations among the various groups.

As in the preceding series, the ash content of the femurs was highest for those rats fed on the maize which had been germinated 96 hours and autolysed for 10 days. Although the autolysed product had been fed at a level considerably lower than in the preceding series, the femurs contained 46.8 % ash. The control ration of the cooked maize produced femurs containing 39.3 % ash. These figures are respectively 17.5 % and 10.0 % higher than those obtained with the germinated maize (29.3 %); but only 11.6 and 4.1 % higher than values obtained with the untreated maize (35.2 %). Moreover when consideration is given to the fact that the value obtained with germinated maize is 5.9 % lower than that obtained with untreated maize, the significance of the difference in calcification produced by the cooked maize, if any, becomes questionable. On the other hand, the values obtained with the autolysed product indicate the formation of distinctly better calcified bone even when these relations are taken into account. The percentage of ash (46.8 %) obtained with the autolysed maize also compares favourably with the 56.7 % obtained in the preceding series when allowance is made for the difference in levels of this constituent in the two rations, 49 % in the former and 76 % in the latter. Sprouting of the maize *per se* gave no

evidence of increasing its antirachitic activity; in fact the percentage of femur ash was actually lowered in this series. Such a failure to demonstrate any change in antirachitic activity due to the process of germination is in accord with the results obtained in the earlier test. The slight increase in calcification observed when whole kernel maize soaked for 16 hours was fed in the preceding series of experiments was not substantiated in this series. In fact the 33.3 % ash obtained was nearly 2 % below that found with untreated maize. Neither did heating the ground maize for 24 hours produce any significant alteration from the result obtained with the untreated maize. The percentages of ash were 31.7 and 35.2 % respectively.

These relations, based upon a comparison of the ash content of the femurs, were substantiated not only by the average weights of the extracted femurs and the average weights of ash but also by the ranges of individual values from which these averages were calculated. Confirmation was also obtained from the width of the uncalcified metaphyses observed in the silver nitrate staining test and to a lesser degree by the extent of involvement of the costochondral junctions.

Series III. Further tests of the effect of heat and moisture upon ungerminated and germinated maize were necessary to interpret properly the results already obtained. To this end the following maize products were prepared: (1) untreated; (2) ground, moistened, dried 27 hours; (3) ground, moistened, dried 10 hours; remoistened, dried 17 hours; (4) germinated 96 hours, dried 27 hours; (5) germinated 96 hours, dried 10 hours; remoistened, dried 17 hours; (6) germinated 96 hours, dried 2 weeks.

It was necessary to determine if heat and the presence of moisture had a different effect upon the germinated maize from that which they had on the untreated seed. Furthermore, it was possible that heat functioning in the presence of moisture might have an effect different from that in the absence of moisture.

The ground products were spread loosely in granite-ware pans in a layer of about a half an inch in depth. The conditions of drying approximated to those used in the process of desiccating former preparations. For the first 10 hours the temperature was held between 47 and 50°, during the following 12 hours the lowest point reached was 33°; throughout the final 5-hour period it was again maintained between 47 and 50°. Moisture additions were made in amounts barely sufficient to cover the ground corn and the pans were immediately placed in front of the fan.

As in the former series these maize preparations were fed as the cereal portion of Ration 2965 in the same manner as described for Series III in a previous publication [Templin and Steenbock, 1933].

Six rats from as many litters and ranging in age from 23 to 30 days were placed on each ration for a five-week feeding period in which the food consumption of all rats was maintained at the same level.

The data are presented collectively in Table III. Regardless of whether or not the maize had been germinated or subjected to varying types of heat and moisture treatment below a temperature of 50°, all rats became very rachitic. The untreated maize produced femurs containing 32.7 % of ash. After moistening and drying, this same maize produced femurs with 31.5 and 33.3 % ash content. The ash contents of femurs produced on the ration containing germinated corn subjected to 27 hours of drying were 27.1 and 30.5 %. An extension of the time of drying to 2 weeks did not alter the rachitogenic property of the germinated maize, for the ash content of the femurs was 30.8 %. These same relations were found for the weights of extracted femurs and the weights of

Table III. *Effect of heat treatment and germination of maize upon calcification.*

Ration	Body weight		Average daily food consumption g.	Blood-serum analyses		Femur data			Maximum width of metaphyses cm.	Costochondral junctions
	Initial g.	Gain g.		Ca mg./100 cc.	P mg./100 cc.	Weight of extracted bone g.	Weight of ash g.	% of ash		
R 91. Untreated	61	44	6.9	13.6	3.5	0.1085	0.0355	32.7	0.17	Much enlarged, some angulation and double beading
R 92. Ground, moistened, dried 27 hours	60	43	6.9	12.8	4.6	0.1081	0.0341	31.5	0.18	"
R 93. Ground, moistened, dried 10 hours; remoistened, dried 17 hours	59	45	6.9	11.0	4.2	0.1095	0.0365	33.3	0.18	"
R 94. Germinated 96 hours, dried 27 hours	61	34	6.8	11.6	4.0	0.0994	0.0269	27.1	0.18	Much enlarged, severe angulation, triple beading
R 95. Germinated 96 hours, dried 10 hours; remoistened, dried 17 hours	61	33	6.8	—	—	0.1001	0.0307	30.5	0.19	Much enlarged, moderate angulation and some double beading
R 96. Germinated 96 hours, dried 2 weeks	59	36	6.9	11.6	4.1	0.1011	0.0312	30.8	0.17	Much enlarged, moderate angulation, double beading

femur ash. Confirmation of the very rachitic condition of all the groups of animals was obtained in the low level of blood-serum-phosphorus, in the presence of wide rachitic metaphyses in the wrist bones and in the manifestations of severe involvement of the costochondral junctions.

Good growth was obtained on an average food intake of 6.9 g. per rat per day. The fact that the average weight gains made by rats on the ungerminated maize were 9 g. greater than on the germinated maize is not, in our opinion, a complicating factor in the interpretation of the results of this series. Neither is it significant in itself since such a difference was not uniformly obtained in the previous tests.

The results of this series again demonstrated that the germination of maize did not alter its rachitogenic property. Furthermore the heat treatment incident to the process of drying the maize products was not responsible for the variations in calcification observed with immature maize.

DISCUSSION.

It is probably premature to attempt to give an explanation of the results obtained by Schittenhelm and his co-workers in the light of our results. Whereas our studies dealt with maize, the German workers fed only dried barley sprouts or extracts thereof. Mellanby *et al.* [1928; 1929] postulated that vitamin D may be formed from ergosterol by the growing plant independently of ultra-violet radiations. De Ruyter de Wildt and Brouwer [1932] reported that barley meal prepared from the entire grain contains vitamin D, whereas that prepared from maize does not. Proof of the above statements is not at present forthcoming and other possibilities can be entertained. For instance, since calculations based on data reported by Täufel and Rusch [1929] indicate that the weight of dry barley sprouts equals less than 4 % of the weight of the barley from which they are formed, it is possible that the Schittenhelm group of workers fed more concentrated products than investigators who used the entire grain and sprouts or even extracts prepared from them. Perhaps the specific rachitogenic factor

referred to by Mellanby [1925] was retained in the grain, thus allowing the nutrients in the sprouts to function without the presence of such antagonistic effects; or the rachitogenic factor may have been destroyed during the progress of germination. Again, the conversion of phosphorus compounds into forms more available to the growing animal would not be revealed by ordinary analysis but would have the same effect as an increase in the phosphorus of the ration. Some of these considerations apply equally well to the interpretation of the results reported here in connection with germinated and autolysed maize.

SUMMARY.

Yellow maize germinated for 95 hours, with roots not exceeding 4 inches in length, was found as rachitogenic as ungerminated maize.

Germinated maize dried below 50° had practically the same rachitogenic properties as the maize from which it had been prepared.

Autolysed germinated maize was definitely less rachitogenic than germinated or untreated maize.

The antirachitic effect of autolysed germinated maize was most pronounced in maize which had been germinated for a long period of time.

Soaked whole kernels and moistened ground maize dried by the same methods as those employed for the sprouted products did not differ from untreated maize in rachitogenic properties.

Heat treatment of ground maize at a temperature less than 50° did not alter its rickets-producing tendency, neither did germinated maize subjected to this temperature for 2 weeks undergo a change in this property.

The authors wish to express their appreciation to the Quaker Oats Company whose financial support made this research possible. The authors are also indebted to Mr James T. Lowe and Mr Robert W. Haman for assistance in conducting blood analyses.

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